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Inside a tract of ice stranded as Greenland's Helheim Glacier retreated, meltwater cascades near Greg Hanlon, an engineer with the U.S. Army Corps of Engineers. Hanlon is part of a team studying how meltwater interacts

with warm ocean currents to speed ice loss from Helheim and other glaciers draining Greenland's ice sheet. See page 170. *Photo: Adam LeWinter, U.S. Army Engineer Research and Development Center (ERDC) Cold Regions Research & Engineering Laboratory, Remote Sensing/GIS Center of Expertise*

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SCIENCE JAPAN

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Responsible genetic genealogy

The scientific development of forensic genetic genealogy (FGG), which couples genetic analysis with investigation of publicly available genealogy information, has successfully transformed law enforcement investigations by solving more than 50 cases over the last 18 months in the United States. However, use of FGG by law enforcement has preceded widespread development of best practices to protect the genetic privacy of private citizens who have voluntarily submitted samples to genealogy databases. Absent best practices, use of FGG could lead to compromised cases, diminished use, or the loss of this new investigative tool. Public support for FGG could be jeopardized and confidence in forensic DNA analysis could be undermined. As the custodian of a national law enforcement DNA database (CODIS), the U.S. Federal Bureau of Investigation (FBI) is looked to by many in the law enforcement and forensic DNA communities for guidance, and its efforts often influence the global community. The emergence of FGG suggests that further discussions on privacy, genomics, and the use of genealogy by law enforcement would be beneficial. Accordingly, the FBI seeks to engage the scientific and bioethics communities in such a dialogue.

Use of FGG involves databases and family trees composed of genetic data of private citizens who are not under suspicion for any crimes. When searching crime scene DNA in these databases, potential perpetrators may be uncovered by identifying their close or distant relatives, and then building family trees that can extend over many generations and may include hundreds to thousands of relatives. To date, this approach is only used if crime scene DNA has not matched genetic profiles in the CODIS database of known offenders and arrestees. A consensus has emerged that there is no legal prohibition on such use. The question is how it should be done.

Under a recently released interim policy from the U.S. Department of Justice (DOJ), effective this November, federal investigative agencies may develop internal policies and procedures and can utilize FGG if the case involves an unsolved violent crime (homicide

or sex crime) for which a CODIS search resulted in no matches, and for which reasonable investigative leads have been pursued. The DOJ Interim Policy is the first substantial attempt to address “how genetic genealogy should be done.” The interim guidance restricts investigative agencies to using only public databases or direct-to-consumer genetic genealogy services that provide clear notice to users and the public that law enforcement may access their sites for investigative or unidentified human remains identification purposes.*

The forensic DNA community is also working on guidance to address the “how to” question. In April, the Scientific Working Group on DNA Analysis Methods (SWGDM; swgdam.org), which recommends standards to the FBI for CODIS and issues guidance for the forensic DNA community, formed an interim committee on FGG composed of genealogists, bioethicists, academicians, law enforcement, and forensic scientists, as well as representatives of the European Network of Forensic Science Institutes and the International Society of Forensic Genetics (the author is co-chair of this committee). This group held an FGG technical symposium for SWGDAM membership in July and recently submitted recommendations to SWGDAM leadership that included establishing an FGG Working Group.

With the FBI and other agencies now moving to develop internal policies and procedures under the DOJ interim policy, the FBI has committed to leading the process of receiving stakeholder input by hosting a symposium on Genetic Privacy and Law Enforcement in 2020. In addition to symposium presentations, a comprehensive discussion of the interim FGG policy should also consider comments solicited from the scientific community on FGG privacy and ethical implications, metrics required by the interim policy, transparency, and SWGDAM guidance and recommendations. To initiate this review, the scientific community and other interested parties are encouraged to provide the FBI with comments at forensicgenealogy@fbi.gov.

—Thomas F. Callaghan



Thomas F. Callaghan is chief biometric scientist at the U.S. Federal Bureau of Investigation (FBI) Laboratory Division in Quantico, VA, USA. tfcallaghan2@fbi.gov

“Absent best practices... confidence in forensic DNA analysis could be undermined.”

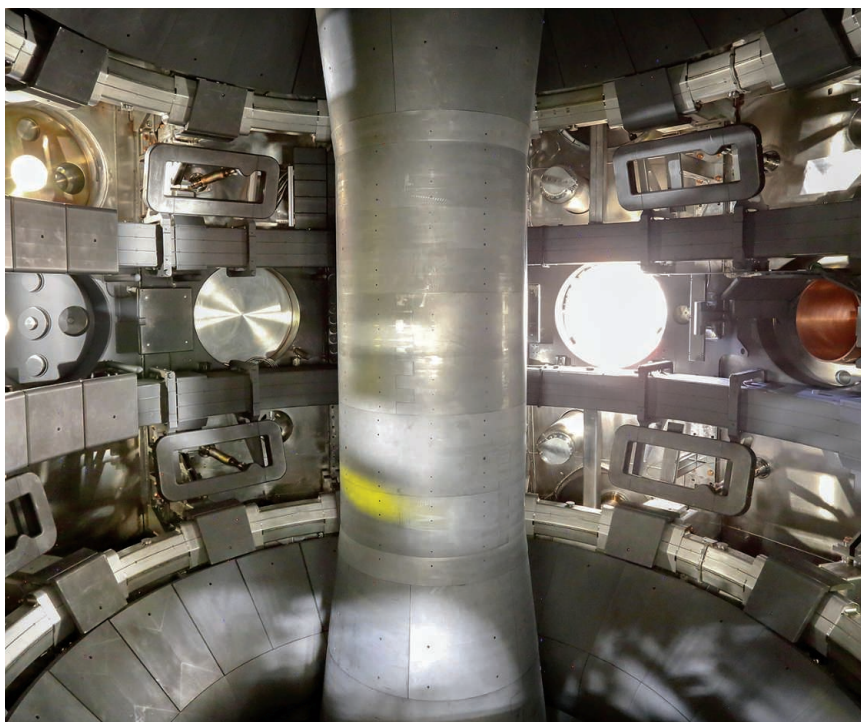
*U.S. Department of Justice, Interim Policy on Forensic Genetic Genealogical DNA Analysis and Searching (2019); www.justice.gov/olp/page/file/1204386/download.

“We’ve found our home for the months to come.”

Markus Rex, leader of the MOSAiC expedition, after its ship stationed itself last week along an Arctic ice floe, where it will be frozen in place for 1 year of climate research.

IN BRIEF

Edited by **Jeffrey Brainard**



The Mega Ampere Spherical Tokamak could pave the way for a next-generation fusion reactor.

PHYSICS

U.K. plans prototype fusion power plant

With the United Kingdom’s future as a research partner in Europe’s fusion program still in doubt ahead of the Brexit deadline this month, the government last week announced it will strike out alone with a £220 million effort to design a next-generation fusion reactor. The Spherical Tokamak for Energy Production (STEP) is meant to be a prototype, commercially viable power plant that could be up and running by 2040. Most fusion researchers around the world are focused on ITER, a giant reactor in France due to fire up in 2025. ITER’s design is called a tokamak, a doughnut-shaped vessel in which hydrogen isotopes are heated to enormous temperatures until they fuse, releasing heat. The Culham Centre for Fusion Energy in Abingdon, U.K., which will host STEP, has pioneered the spherical tokamak design, which is shaped like a cored apple and is simpler than ITER’s. Culham’s existing spherical tokamak, the Mega Ampere Spherical Tokamak, will serve as a testbed for STEP.

Harassment spurs MIT firing

#METOO | The Massachusetts Institute of Technology’s troubled Media Lab fired a veteran scientist on 26 September for sexual harassment. Michael Bove, who headed the Object-Based Media Group in Cambridge, apologized in a statement sent to Media Lab students, staff, and faculty on 3 October. At a staff meeting on 27 September, Media Lab leaders announced mandatory sexual harassment training for its personnel. The lab’s former chief, Joi Ito, resigned on 7 September in the wake of a news report that he concealed the extent of the Media Lab’s relationship with donor Jeffrey Epstein, a convicted sex offender who died by suicide in a New York City jail in August.

Societies pull BYU job ads

WORKPLACE | After a public outcry, the American Geophysical Union (AGU) and the Geological Society of America (GSA) last week removed from their websites job ads by Brigham Young University (BYU) in Provo, Utah, because the institution’s Honor Code bars employing people who engage in “homosexual behavior.” Both AGU, based in Washington, D.C., and GSA, in Boulder, Colorado, acted after members tweeted that the ads violated the societies’ policies against discrimination. The organizations have policies banning ads that mention discriminatory employment practices. BYU’s job postings mentioned its Honor Code, but not the details of its requirements. Both societies told *Science* they had never removed an ad under similar circumstances before; GSA said it began to review its ad policies after last week’s removal. AAAS, publisher of *Science* in Washington, D.C., says it also examines ad content, and if complaints arise it reviews job advertisers’ employment policies for consistency with its own policies against discrimination.

Accelerator budget proposed

PARTICLE PHYSICS | The International Linear Collider—a next-generation accelerator intended to study the Higgs boson, an essential but little understood component of the Standard Model of particle physics—advanced a step last week with a cost-



MARINE ECOLOGY

Lots of extra genes explain this invasive mussel's success

Both accidentally and deliberately, people have spread the Mediterranean mussel (*Mytilus galloprovincialis*, above) to locales around the world, where it is used in aquaculture and often displaces native species. Marine biologist Antonio Figueras at the Institute for Marine Research in Vigo, Spain, and colleagues now say this mollusk's success is likely explained by its large, flexible, and unusual "pangenome"—the animal has a core

genome of 45,000 genes, but any individual can have up to 15,000 additional protein-coding, dispensable genes, some of which can improve survival, the researchers reported on 25 September in a bioRxiv preprint. It's the first pangenome discovered in an animal. Figueras says it may give the immobile mussel an edge because, "like in bacteria, fungi, and plants, the high prevalence of dispensable genes might represent an invaluable adaptive resource."

sharing proposal released by an international working group. Japan has been a front-runner to host the facility, which would smash electrons with positrons, but the government has deferred a decision because of concerns over its share of the estimated \$7 billion cost, not including detectors. The working group, organized by Japan's High Energy Accelerator Research Organization, suggested that the host country, presumably Japan, cover the tunnels and buildings, about 22% of the project's total cost. Japan and other participating countries would then make in-kind contributions for accelerator components, 63% of the total budget, and share additional costs for installing electrical and water lines. Japan's government has not yet reacted to the cost proposal.

Japan to hunt gravitational waves

ASTROPHYSICS | A fourth detector has joined the international network hunting for gravitational waves—ripples in space created, for example, when two black holes crash together. Last week, physicists with the new Kamioka Gravitational Wave Detector (KAGRA) in central Japan inked a data-sharing agreement with researchers operating similar instruments—the Laser Interferometer Gravitational-Wave Observatory, comprising a pair of detectors in the United States; and Virgo, a detector near Pisa, Italy. A

fourth detector will help the network better pinpoint sources in the sky. KAGRA employs next-generation features, such as mirrors cooled to -253°C to limit vibration-induced noise in the data. KAGRA physicists hope to start to take data this year, but it may take months or even years to tune the device well enough to detect signals.

Saturn leads in moon count

PLANETARY SCIENCE | The solar system has a new moon king. Astronomers have discovered 20 new, small moons orbiting Saturn, giving the gas giant a total of 82 moons, just edging out Jupiter's 79, a team led by Scott Sheppard at the Carnegie Institution for Science in Washington, D.C., announced this week. The new moons are tiny, each about 5 kilometers in diameter, and most orbit in a direction opposite to the planet's rotation. Sheppard's team, which also discovered many of Jupiter's moons, is holding a contest to name Saturn's new satellites; proposed names must be drawn from Norse, Gallic, or Inuit mythology.

BioRxiv posts peer reviews

PUBLISHING | BioRxiv, the server for life sciences preprints, announced last week that it has begun an experiment allowing journals and independent peer-review services to post evaluations of its papers. About two-thirds of the papers posted on

bioRxiv are eventually published in peer-reviewed journals, but the published papers rarely include referees' reports. Even if they do, readers see the paper's final version, not the draft reviewed. Pairing journals' reviews with the preprint version will increase transparency, says Richard Sever, co-founder of bioRxiv, based at Cold Spring Harbor Laboratory in New York. Authors will be required to opt in to the posting of reviews, regardless of whether they are favorable. The experiment's initial participants include two publishers, eLife in Cambridge, U.K., and EMBO Press in Heidelberg, Germany, and two independent peer-review services, Review Commons and Peerage of Science. Posting the services' reviews could provide a public quality check for preprints never published in journals, Sever says.

Warbler endangered no more

CONSERVATION | The U.S. Fish and Wildlife Service announced on 8 October the removal of Kirtland's warbler from the list of endangered species. The yellow-breasted bird, now found in Michigan, Wisconsin, and Canada, was among the first species to receive federal protection in 1967. A decadeslong conservation effort helped the population rebound from 200 singing males to more than 2300 breeding pairs by 2015, double the original recovery goal. Critics of the Endangered Species Act say once species are listed, they are too rarely removed.



Stephen Thomas (center) is a researcher at the University of Maryland in College Park who helps train barbers to be health advocates for their customers.

RESEARCH FUNDING

Topic choice works against black NIH applicants

Study flags new factor contributing to racial disparity in who wins grants

By **Jeffrey Mervis**

In 2011, a study led by economist Donna Ginther of the University of Kansas in Lawrence found that black applicants were significantly less likely than white applicants to be funded by the National Institutes of Health (NIH). Since then, NIH officials have examined a host of factors that might cause the disparity, from the historical advantages that white men enjoy to overt discrimination by grant reviewers. But the picture remains cloudy.

Now, NIH scientists have identified a key factor they hadn't previously considered: the topics that black scientists want to study. Specifically, black applicants are more likely to propose approaches, such as community interventions, and topics, such as health disparities, adolescent health, and fertility, that receive less competitive scores from reviewers. And a proposal with a poorer score is less likely to be funded. The finding is already prompting discussion about whether that disparity is rooted in NIH's priorities—and whether those priorities should be rethought.

The study, published on 9 October in *Science Advances*, is based on some 157,000 proposals submitted between 2011 and 2015 for NIH's bread-and-butter R01 grants. After analyzing the text, researchers

placed each proposal into one of 150 topic areas. Then they examined six factors that could influence the final outcome. They found that three contributed to creating the "Ginther gap"—whether a proposal is scored (more than half are not), what score it receives, and the applicant's choice of topic.

Among scored proposals, topic choice emerged as the second biggest contributor to the Ginther gap. (It trailed only an applicant's track record, which was the focus of a previous study.) Topic choice accounts for 21% of the overall funding disparity, the researchers found. Three other factors—how frequently scientists send in proposals, how NIH institutes acted on study section recommendations, and whether a scientist revised a rejected proposal and tried again—were found not to contribute to the gap.

The authors reject the idea that an especially "harsh" study section could contribute to the disparity. As many as 49 different panels received proposals within a particular topic area. Similarly, a single panel handled proposals touching on as many as 27 topics, reducing its influence on any given topic. The paper also reminds readers that "numerous analyses have shown" that reviewer scores do not predict the ultimate influence of a particular award, as measured by standard metrics such as the number of citations on papers produced by the grant.

The study takes "a big step" toward understanding where the disparity occurs, says co-author James Anderson, who directs NIH's Division of Program Coordination, Planning, and Strategic Initiatives. But it doesn't answer the question of why reviewers are "less excited about" proposals on topics that disproportionately interest black scientists, he admits. "This study just looked at the numbers. The next step is to ask the people who made the decisions." No such studies are in the works, he adds.

Some researchers not involved in the study speculate that the explanation for the disparity goes beyond possible reviewer bias, to how NIH sets spending priorities and the larger societal factors that drive those decisions. "I think the bias is more structural than racial," says Stephen Thomas, a professor of health services at the University of Maryland in College Park. "It's really a disciplinary bias. The current NIH system favors basic science with no regard for practical applications over research that applies what we already know to address the health crisis facing our country."

Thomas, who directs the NIH-funded Maryland Center for Health Equity, says he's not surprised that many black researchers want to tackle pressing community problems. "As an African American who came up through the academic ranks and

PHOTO: CADE MARTIN

has the scars to prove it, I can understand why someone growing up among people who have been systematically discriminated against may be motivated to become a scientist because of a desire to address those problems,” he says. “I’m not saying that doesn’t motivate white scientists, too. But I’ve seen it in many of my students.”

At the same time, Thomas warns, students pursuing such a path within academia may wind up “getting mentored out” of taking that approach. “They are told it’s not the way to build their career,” says Thomas, who is part of the NIH-funded National Research Mentoring Network, which is working to boost the fortunes of minority scientists.

But mentoring isn’t the only challenge black applicants face. Because almost all reviewers are also NIH grantees, the dearth of black scientists receiving R01 awards—1% of the total in 2018—translates into few black reviewers. That means fewer people who, because of their backgrounds, “might have different opinions on the significance of some grant applications,” the paper notes.

The disparity could also reflect a broader phenomenon in which work by women and minorities is often seen as having lower status, says Molly Carnes, a professor of women’s health at the University of Wisconsin in Madison who has studied implicit bias. “I don’t think that NIH reviewers are consciously saying, ‘This project is more worthy of funding because the proposal is associated with traits seen as high status,’” Carnes says. “It’s just the way people’s minds work.”

Carnes applauds NIH for doing the study “because now [this issue] can be addressed.” One possible remedy, she says, is to redefine some of the topics favored by black scientists in a way that grants them higher status. For example, she notes that a field once called “community-based research” has been renamed “implementation science” to emphasize its focus on moving research findings from the bench to the bedside. A more rigorous study design can also impress reviewers, she adds.

The authors recommend that NIH institute directors consider spending more of their budgets in areas “that are underappreciated by reviewers but that align well with their strategic priorities.” But NIH officials say they need time to digest the results before applying any possible remedies. “The underlying explanation for the phenomenon ... requires further study to determine how best to intervene,” Hannah Valentine, NIH’s head of scientific workforce diversity, wrote in a release accompanying the paper.

Any intervention will need community buy-in, Thomas and Carnes add. “You have to work within the system,” Carnes says, “and mold it to what you want to achieve.” ■

HUMAN RIGHTS

Science organizations speak out in defense of Uyghur academics

Several internationally known researchers have been swept up in massive detentions in China’s Xinjiang province

By Catherine Maticic

No one outside the Chinese government knows where Tashpolat Tiyp is. No one knows exactly what charges have been filed against him. The only thing that anyone really knows is that in April 2017, as the geographer and former president of Xinjiang University in Ürümqi prepared to fly from Beijing to Berlin for a scientific conference and the launch of a research center, he disappeared without even a phone call to colleagues or family.

Six months later, a Chinese propaganda video emerged saying Tiyp was one of 88 scholars who had “deeply poisoned the minds” of students by approving textbooks with too much content from Uyghur sources—the ethnic group that makes up about half of Xinjiang province’s 24 million people. The video calls Tiyp and three other Uyghurs “two-faced” separatists before announcing their sentence: death, with a 2-year reprieve.

“It just doesn’t make any sense to anybody,” says Gary Langham, executive director of the American Association of Geographers (AAG), which last week sent Chinese president Xi Jinping a letter asking him to halt the execution and release Tiyp unless there is evidence he committed actual crimes. It was signed by more than 1300 researchers from 50 countries. (AAG took action after Amnesty International warned that Tiyp’s execution could be imminent.)

China’s crackdown on mostly-Muslim minorities in the far western province of Xinjiang, which include the Turkic-speaking Uyghurs, Kazakhs, and Kyrgyz, has swept up as many as 1 million people. But Tiyp is one its best-known victims.

Scientific organizations outside of China are trying to help him and other internationally known researchers who have disappeared. But many are moving cautiously, worried about making things worse. And it’s unclear whether, in China’s current political climate, such support from abroad makes any difference.

Since late 2016, China has detained as many as one in 11 Uyghurs in Xinjiang, most in grim re-education camps with barbed wire fences and guard towers. There, they learn party slogans and songs, and study the Chinese language. Government officials say the camps are necessary to crack down on 20 years of “violent terrorism” in the region. But human rights

groups say the operation is an attempt to erase Uyghur culture and traditions; some call it “cultural genocide.” (When asked by *Science* why so many have been detained, a Chinese foreign ministry spokesperson did not respond.)

Tiyp’s detention came as a surprise to many colleagues because he was an official in the Chinese Communist Party and a “red person,” someone known for carefully following party rules, says a Uyghur social scientist who knows Tiyp and asked not to be identified.

Tiyp studied in Japan after getting a bachelor’s degree at Xinjiang University, but in 1992, he returned to his alma mater to teach and study desertification and soil salinization using remote sensing technology. His work on arid ecosystems ecology led to an honorary degree from the École Pratique des Hautes Études, part of the Sorbonne in Paris, in 2008. By 2010, he was the university’s president as well as its deputy party secretary.

“They interviewed him on TV a thousand times,” the social scientist says, usu-



Tashpolat Tiyp disappeared in April 2017 while en route to Germany.

ally to showcase Tiyp's rise from a humble farming background to his prominent position in Uyghur—and Chinese—society. “[They would say] he’s a president, a self-made man with party support.” Yet that did not protect him, the scientist adds: “If they took [him] ... there’s no hope for the rest of us.”

Tiyp was apparently replaced as university president in late March, just a few weeks before his arrest. He disappeared en route to Germany, where he was supposed to attend the launch of a joint center to study underground coal fires, a collaboration between Xinjiang University and the Leibniz Institute for Applied Geophysics in Hanover. Tiyp had championed the center as university president.

Other internationally known researchers have been arrested in the crackdown, including Rahile Dawut, an anthropologist at Xinjiang University who studied regional literature and Uyghur oral traditions; Halmurat Ghopur, an official at the Xinjiang Food and Drug Administration and past president of the Xinjiang Medical College Hospital, who has also been sentenced to death for separatism; and Ilham Tohti, an economist sentenced to life in prison in 2014 on charges of separatism who last month won the Václav Havel Human Rights Prize, an annual award from the Parliamentary Assembly of the Council of Europe.

The detentions have put international scientific societies in a difficult position, says Clare Robinson, director of advocacy for the New York City-based group Scholars at Risk, which has repeatedly called for Tiyp's release. AAG is one of several societies that have publicly supported colleagues in Xinjiang. But Robinson says speaking out risks disrupting academic collaborations with China and could lead to visa bans for foreign academics—or put their Chinese colleagues at risk. “You need to pick your public voice carefully,” Robinson says. The private diplomacy of Nobel laureates and university presidents is often more effective, she says. Such behind-the-scenes work is ongoing in Tiyp's case and others.

Robinson says the mass detentions in Xinjiang province are unconscionable, regardless of who's affected. But by targeting academics and intellectuals, authorities are robbing Uyghur—and Chinese—society of an important part of its future, she says. “If you remove from your functioning society all of the future scholars, the current scholars, the scientists,” Robinson says, “you’re losing an entire generation of individuals who could contribute to the production of knowledge.” ■

PHYSICS

Nobel honors pioneers in cosmology and exoplanets

Researchers found “hot Jupiters” around other stars and predicted universe’s chief ingredients

By **Adrian Cho** and **Daniel Clery**

This year’s Nobel Prize in Physics honors the human desire to understand both the fundamental nature of the universe and its details. Half of the \$900,000 prize goes to Princeton University cosmologist James Peebles, for laying the foundations of modern-day cosmology. The other half is split between astronomers Michel Mayor and Didier Queloz. In 1995, at the University of Geneva in Switzerland, they discovered the first planet around another sunlike star—opening the floodgates for the discovery of thousands more exoplanets of every description.

Peebles’s many theoretical predictions have proved prescient. He “put the physics into cosmology,” says Joseph Silk, a cosmologist at the University of Oxford in the United Kingdom. For starters, in 1965 he predicted that the big bang should have left an afterglow; the cosmic microwave background (CMB) was discovered the same year.

Peebles also predicted that CMB temperatures would vary from point to point because of the sloshing of radiation, ordinary matter, and dark matter—the invisible stuff thought to hold the galaxies together. Those fluctuations were eventually spotted by NASA’s Cosmic Background Explorer satellite, launched in 1989.

To get the size of those fluctuations right, Peebles had to take into account not only dark matter, but also a kind of dark energy in empty space. Sure enough, in 1998 astronomers discovered that the expansion of the universe is accelerating, driven by dark energy. Building on Peebles’s framework, CMB studies now peg the universe at 5% ordinary matter, 26% dark matter, and 69% dark energy. Chuck Bennett, a cosmologist at Johns Hopkins University in Baltimore, Maryland, notes that Peebles’s prize is “more a lifetime achievement award than an award for one master stroke.”

Mayor and Queloz, in contrast, changed astronomy with a master stroke—though even they wondered at first whether their

1995 discovery could be real. They had found a gas giant half the mass of Jupiter in an orbit much tighter than Mercury’s. “They took a long time to convince themselves,” says astronomer Nikku Madhusudhan of the University of Cambridge in the United Kingdom.

Now, a quarter-century later, exoplanets have gone “from obscure, fringe, and laughable to Nobel-worthy,” says astronomer Sara Seager of the Massachusetts Institute of Technology in Cambridge.

In the 1980s, after years of searching for exoplanets, astronomers adopted a new, indirect technique: watching starlight. The gravity of a large planet, as it orbits, can tug its star around. Astronomers can thus look for periodic Doppler shifts in the wavelengths of the star’s light as

it moves to and from Earth.

In April 1994, Mayor and Queloz began to watch 142 sunlike stars for wobbles with a new spectrometer at the Haute-Provence Observatory in France. One star, 51 Pegasi, offered a clear signal. From the size and timing of the oscillation and the mass of the star they calculated that the planet, a gas giant called 51 Pegasi b, was a “hot Jupiter.” It suggested that giant planets may form far from their stars and then migrate inward.

In 2000, a new way to catch exoplanets emerged: watching for periodic dimmings in starlight as a planet passes in front, or transits. Whereas wobbles have revealed several hundred exoplanets, the transit method has bagged several thousand.

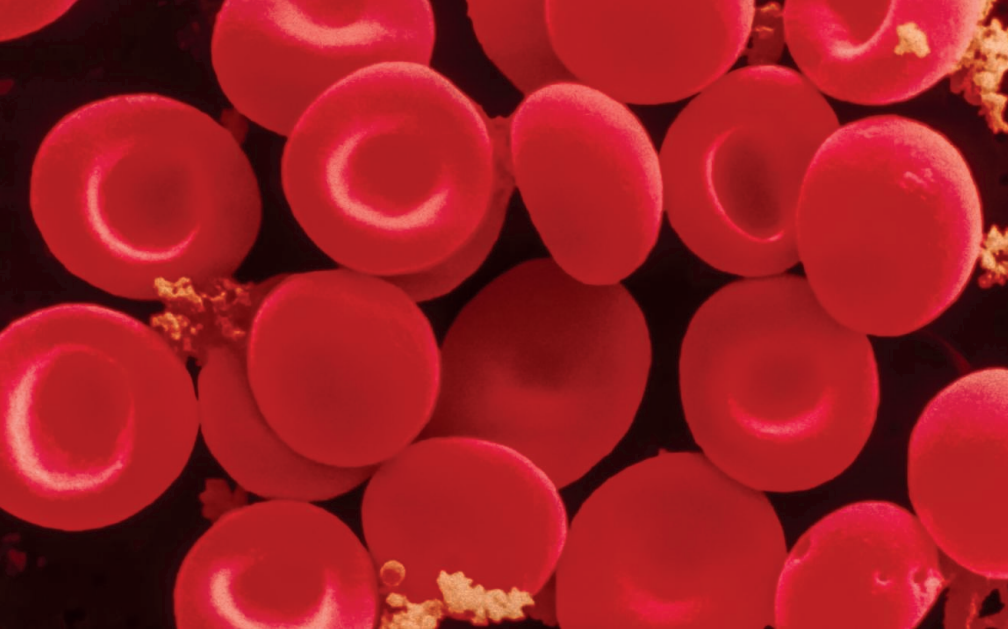
Mayor, now retired, and his team discovered about 200 more exoplanets. Queloz, now at Cambridge, is involved in a project dubbed SPECULOOS to detect planets around the smallest stars using the Doppler method. According to its chief, Michaël Gillon of the University of Liège in Belgium, the call from the Nobel Committee came when Queloz was in a project meeting Tuesday morning. “It’s really well deserved,” says Gillon, who did a post-doc fellowship in Geneva with Mayor and Queloz. “The next decade will be super exciting for the field.” ■

PHYSICS

“For theoretical discoveries in physical cosmology”
James Peebles

“For the discovery of an exoplanet orbiting a solar-type star”

Michel Mayor
Didier Queloz



Cells that sense low oxygen turn on many genes, including one that boosts red blood cell production.

BIOMEDICINE

Cellular oxygen sensor system earns Nobel for trio

Discoveries led to candidate drugs for anemia and cancer

By Kai Kupferschmidt

Cregg Semenza's "really rough year"—he's recovering from a fall that broke several neck vertebrae—just got a whole lot better. The physician-scientist at Johns Hopkins University School of Medicine in Baltimore, Maryland, and two other researchers this week shared the Nobel Prize in Physiology or Medicine for their studies, mostly in the 1990s, that uncovered how cells in the human body respond to low oxygen levels.

The prize-winning research, by Semenza, William Kaelin Jr. at the Dana-Farber Cancer Institute in Boston, and Peter Ratcliffe of the University of Oxford in the United Kingdom, has far-reaching implications for medicine, says cellular biologist Celeste Simon of the University of Pennsylvania Perelman School of Medicine, another leader in the field. "Oxygen limitation is a part of virtually all diseases, not just solid tumors or stroke, but inflammation, wound healing, peripheral arterial disease. All of these involve decreased oxygen."

As a result, the trio's work promises powerful new treatments for diseases including anemia and cancer, some already in clinical trials around the world. "How oxygen is sensed by both normal tissues and tumors is an incredibly important dis-

covery that is highly deserving of a Nobel Prize," says Amato Giaccia, a cancer researcher at Oxford.

In the 1980s, biologists already knew that low oxygen levels in the body increase transcription of the gene for the hormone erythropoietin (EPO) in the kidney, which in turn boosts production of oxygen-carrying red blood cells. That response helps ensure that tissues get enough oxygen. But how the body senses a lack of oxygen was unclear.

In 1992, Semenza pinpointed a key protein, which he called hypoxia-inducible factor (HIF). It binds to DNA regulating multiple genes, including EPO's, that help the body cope with low oxygen. Meanwhile, Kaelin entered

the oxygen sensing story while studying an inherited syndrome, von Hippel-Lindau disease, which increases the risks of certain cancers. It's caused by mutations in the gene for a protein dubbed VHL, and Kaelin found that cancer cells lacking the protein had hypoxia-related genes permanently switched on, which can nourish tumors by promoting the growth or remodeling of blood vessels.

In independent lines of research, he and Ratcliffe next worked out how HIF and VHL interact. At normal oxygen levels, enzymes called prolyl hydroxylases (PHDs) chemically modify HIF-1alpha, a subunit of HIF. The modified protein then binds to VHL, creating a complex that is tagged for de-

struction in the cell's garbage disposal, the proteasome. When oxygen is scarce, the PHDs become inactive. That leaves HIF-1alpha free to migrate to the cell's nucleus, where it unites with the other HIF component, HIF-1beta, and alters activity of hundreds of genes, including the one for EPO.

People suffering from renal anemia could be the first to benefit from the trio's research. In these patients, kidney disease leads to low EPO production, which is usually treated with injections of iron supplements and a recombinant form of the hormone; the market for such EPO treatments is billions of dollars every year. But recombinant EPO has side effects, including a higher risk of a stroke or a heart attack.

The Nobel work offers another path: using an oral drug to inhibit the PHDs, the enzymes that lead to HIF being destroyed. Several such drug candidates have been developed, and in 2018 China approved one, roxadustat, made by AstraZeneca. (Even before that, two professional cyclists were suspended for using the substance to boost performance.) Bayer, GlaxoSmithKline, and Japanese pharma company Otsuka also each have a PHD inhibitor in advanced clinical trials of anemia patients.

Whether such drugs have fewer cardiovascular side effects than EPO treatments isn't clear, although a detailed safety analysis of roxadustat is expected to be announced next month at a major nephrology meeting. And the new HIF-preserving drugs bring their own worry: an increased risk of cancer. As in von Hippel-Lindau disease, genes turned on in low-oxygen conditions may also help cancers grow.

Drugs that have the opposite effect—shutting down the hypoxia pathways—could treat cancer. Earlier this year, Merck paid more than \$1 billion to acquire Peloton Therapeutics, which has been developing an inhibitor of HIF-2alpha, a close relative of HIF-1alpha. Clinical trials of its lead compound, PT-2977, in kidney cancer patients are encouraging, Giaccia says. But, here, as well, the worry about side effects persists.

"There's HIF in virtually every cell of the body, in the nervous system, in the blood vessels, the immune system," says Randall Johnson, who studies hypoxia at the University of Cambridge in the United Kingdom and was on the Nobel Prize committee. That ubiquity means that manipulating HIF will have numerous effects, some of which may be harmful. "These drugs that influence the hypoxia pathway will be very powerful, but they will also have to be looked at very carefully," Johnson says. ■

PHYSIOLOGY

"For their discoveries of how cells sense and adapt to oxygen availability"

William Kaelin Jr.
Peter Ratcliffe
Gregg Semenza



In Bronze Age Germany, women traveled far from their family of origin to marry; adult sons stayed at home.

ARCHAEOLOGY

Bronze Age inequality and family life revealed in powerful study

Combined methods show women married far from home

By Ann Gibbons

Four thousand years ago, the Early Bronze Age farmers of southern Germany had no Homer to chronicle their marriages, travails, and family fortunes. But a detailed picture of their social structure has now emerged from a remarkable new study. By combining evidence from DNA, artifacts, and chemical clues in teeth, an interdisciplinary team unraveled relationships and inheritance patterns in several generations of high-ranking families buried in cemeteries on their farmsteads.

Among the most striking of the findings, reported online this week in *Science*, was an absence: “We were totally missing adult daughters,” says team member Alissa Mittnik, a postdoc at Harvard Medical School in Boston. Sons, in contrast, put down roots on their parents’ land and kept wealth in the family.

“What shocked me was that you have to give away all your daughters at some moment,” says co-author Philipp Stockhammer, an archaeologist at Ludwig Maximilian University in Munich and the Max Planck Institute (MPI) for the Science of Human History in Jena, both in Germany. That poignant glimpse into an ancient culture “could not possibly be recovered ... through any one of these methodologies” alone, says historian Patrick Geary of Princeton

University, who was not part of the team.

The researchers worked with remains and grave goods excavated more than 20 years ago, when land along the Lech River south of Augsburg was dug up to build a housing development. Radiocarbon dates showed the farmers lived between 4750 years ago and 3300 years ago. Mittnik was working in the lab of Johannes Krause at MPI, and she and her colleagues analyzed DNA across the genomes of 104 people buried on the farmsteads. The team sought clues to the farmers’ sex and how they were related to one another. The researchers recalibrated the radiocarbon dates, constraining them to within 200 years in some cases, and identifying four to five generations of ancestors and descendants who lived in that time window.

Some of the early farmers studied were part of the Neolithic Bell Beaker culture, named for the shape of their pots. Later generations of Bronze Age men who retained Bell Beaker DNA were high-ranking, buried with bronze and copper daggers, axes, and chisels. Those men carried a Y chromosome variant that is still common today in Europe. In contrast, low-ranking men without grave goods had different Y chromosomes, showing a different ancestry on their fathers’ side, and suggesting that men with Bell Beaker ancestry were richer and had more sons, whose genes persist to the present.

One-third of the women were also buried

with great wealth—elaborate copper head-dresses, thick bronze leg rings, and decorated copper pins. They were outsiders, however. Their DNA set them apart from others in the burials, and strontium isotopes in their teeth, which reflect minerals in the water they drank, show they were born and lived until adolescence far from the Lech River. Some of their grave goods—perhaps keepsakes from their early lives—link them to the Únětice culture, known for distinctive metal objects, at least 350 kilometers east in what is now eastern Germany and the Czech Republic.

There was no sign of these women’s daughters in the burials, suggesting they, too, were sent away for marriage, in a pattern that persisted for 700 years. The only local women were girls from high-status families who died before ages 15 to 17, and poor, unrelated women without grave goods, probably servants, Mittnik says. Strontium levels from three men, in contrast, showed that although they had left the valley as teens, they returned as adults. That “opens a new window into male life cycles,” Geary says.

Bronze Age princely burials have long signaled social inequality. But the organization of these societies remained “rather vague,” Stockhammer says. By combining archaeology with DNA data on family ties, the new study sharpened the picture. The data show, for example, that brothers were buried with equally rich grave goods, indicating that all sons, not just the eldest, inherited wealth. Related men kept wealth in the family for four to five generations.

The burials of poor, unrelated people on the same plot suggested inequality thrived within these households. Such complex social structure in these rather modest farmsteads surprised Stockhammer, who says the archaeological record in Europe first shows servants or enslaved people living under the same roof as higher-ranking people 1500 years later, in classical Greece.

Some researchers hope the same barrage of methods will be applied to other sites. Archaeologist Eszter Bánffy, director of the Romano-Germanic Commission of the German Archaeological Institute in Frankfurt, is excited about the results but notes they only provide “narratives for one region and one period. If similar analyses happen widely in time and space,” researchers can draw more general conclusions, she says.

“While archaeology has provided the bone structure, archaeogenetics has added the flesh,” adds archaeologist Detlef Gronenborn of Johannes Gutenberg University in Mainz, Germany. “The full fascination only emerges when both disciplines are combined.” ■

ILLUSTRATION © TOM BJÖRKLUND

ASTRONOMY

Telescope seeks clues to exoplanet interiors

NenuFAR array looks for radio signals whipped up by the magnetic fields of distant worlds

By Daniel Clery

Astronomers have discovered several thousand exoplanets, but beyond their size, mass, and—for a precious few—a hint of what's in their upper atmospheres, they remain shrouded in mystery. But last week in France, observers unveiled a radio telescope that could reveal what is going on inside an exoplanet. The telescope, tuned to search for beamlike radio signals whipped up by a magnetic field, could show whether a planet has a magnetic dynamo—a churning, liquid metallic core like Earth's.

"It's a probe into internal structure that there is no other way to get at right now," says astrophysicist Evgenya Shkolnik of Arizona State University in Tempe, who is not involved in the project.

What the telescope finds could help researchers understand planet formation and whether the six planets with magnetic fields in our solar system are typical. The signals would also be clues to a planet's habitability. Magnetic fields protect a planet's surface from cosmic rays and the wind of charged particles from its star, which can harm life. By deflecting stellar wind, a magnetic field could also prevent the particles from scouring away a planet's life-nurturing atmosphere. "This opens up an extra door to study exoplanets at a distance," says Jean-Mathias Griessmeier of the University of Orléans in France.

Officially inaugurated last week, the telescope will be a station within the Low Frequency Array (LOFAR), a European radio array centered in the Netherlands. Sited at the Nançay Radioastronomy Station in France, the New Extension in Nançay Upgrading LOFAR (NenuFAR), as the instrument is called, will aid in LOFAR's quest to find signals from the early universe's first stars (*Science*, 7 November 2014, p. 688). But it will also devote a large fraction of its time to scanning a range of radio frequencies for signs of exoplanetary magnetic fields. "It's only a matter of time [before a detection], probably months," Shkolnik predicts.

In the mid-1950s, astronomers first detected radio bursts from Jupiter. Ions that escape from its volcanic moon Io get swept up by the planet's magnetic field and gyrate around the field lines. The whirling ions emit radio photons, which prompt other ions, gy-

rating in concert, to spawn more photons, resulting in a coherent beam, like a natural laser. Later, detectors in space picked up weaker and lower frequency radio signals from other planets, driven by solar wind particles caught in their magnetic fields.

Even Jupiter's strong signal is too weak to be seen at light-year distances. But many of the exoplanets detected so far are "hot Jupiters," gas giants that orbit their stars more closely than Mercury does the sun. Such a planet would be buffeted by a stronger stellar wind—offering more electrons to be whipped up by the planet's magnetosphere into a signal that could be a million times stronger than Jupiter's. In theory, a beam that powerful could be detected from Earth.

Arrays like LOFAR have found suggestive signals before, but nothing certain. NenuFAR, more sensitive at low frequencies and

funding. So far, it has secured 80% of the €15 million needed to build and operate the array, from government funders, universities, and local authorities.

The NenuFAR team will soon devote days-long observing runs to each of a dozen or so nearby hot Jupiters, waiting for a beam of radio energy to sweep past Earth. Other observatories are joining the hunt. The Owens Valley Long Wavelength Array in California will have 352 antennas when it is complete next year. It isn't as sensitive as NenuFAR, so rather than focusing on known exoplanets, it will observe the whole sky continuously. With luck, it will catch the rare, extra bright radio flash of a planet struck by a coronal mass ejection—a fast-moving bubble of stellar wind—says principal investigator Gregg Hallinan of the California Institute of Technology in Pasadena.



The NenuFAR array in France will look for radio signals from hot Jupiters, exoplanets that orbit their star closely.

dedicated to the hunt, may have better luck. It will eventually contain nearly 2000 antennas resembling denuded, wire-frame Christmas trees. Most will sit in a 400-meter-wide core, with a few more widely spread. The receivers pick up frequencies from below 85 megahertz (MHz)—the bottom of the FM radio band—down to 10 MHz, below which the ionosphere blocks any signals from space.

NenuFAR has been gathering data since July with 60% of its antennas working. At the inauguration, the array's principal investigator, Philippe Zarka of the Paris Observatory in Meudon, said he hopes to have 80% of the hardware in place by the end of the year while the team seeks more

Because NenuFAR and other ground-based telescopes have a lower limit of 10 MHz, they will be limited to detecting hot Jupiters—unlikely locales for life. Earth-like exoplanets probably have weaker magnetic fields that produce radio emissions below 10 MHz. To escape the ionosphere that blocks lower frequencies, radio astronomers will have to search from space or the far side of the moon. The latest plan for a lunar radio telescope, known as the Farside Array, is now being considered by the U.S. decadal survey of astrophysics, a priority setting exercise (*Science*, 19 July, p. 234).

Astronomers may have to range far from Earth to find a place like home. ■



OUR FUTURE IN THE FJORDS

How fast will seas rise? A closely observed Greenland glacier could hold an answer

By **Paul Voosen**, in Sermilik Fjord, Greenland

This summer, as meltwater streamed off the Greenland Ice Sheet in record amounts, a ramshackle research ship, the *Adolf Jensen*, sat idling in this fjord, icebergs near its bow and a mystery below it. Two years earlier, oceanographers had moored a sensor in the fjord's depths to decipher how warm Atlantic Ocean waters are eroding Helheim Glacier, one of the ice sheet's largest tongues. But now they couldn't retrieve the 500-meter-deep mooring—or its crucial data.

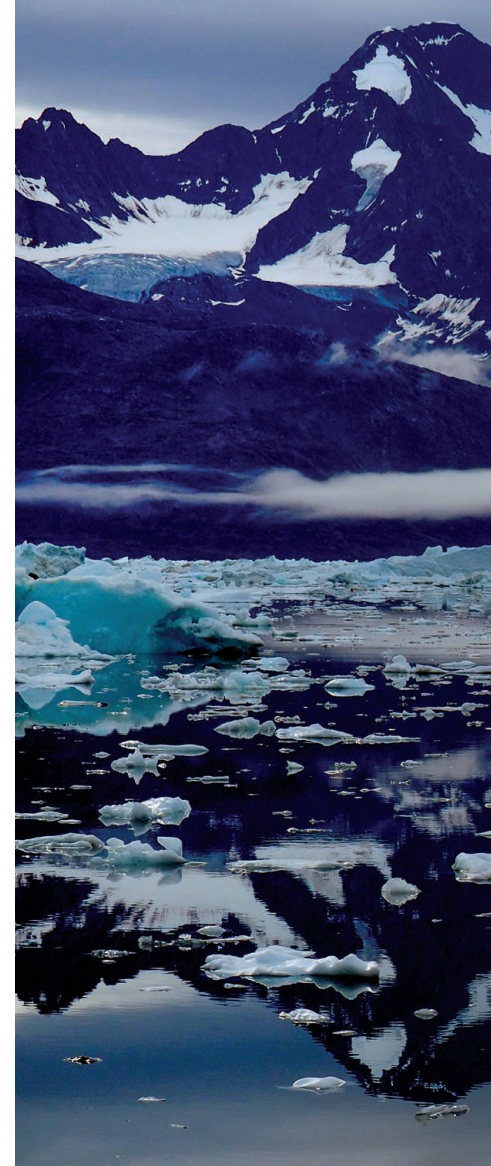
On deck, under a late July sun, Fiamma Straneo, a physical oceanographer at the Scripps Institution of Oceanography in San Diego, California, glared at a yellow box at her feet. It was blasting acoustic chatter at her mooring, telling a latch to let go, but the signal seemed to fall on deaf ears. "Time to come home," she said hopefully. The minutes ticked by and the mooring didn't surface. Straneo grimaced. Over the past decade, she has used such moorings to collect

a long-term record of water masses and currents in Sermilik Fjord, a 90-kilometer-long passage, wider than the Mississippi, that ushers melting icebergs from Helheim out to sea and warm water back in. The stuck mooring threatened to leave a gap.

It was an inauspicious start to an ambitious project. Even after decades of study, researchers can't say how quickly the Greenland Ice Sheet will melt under the strain of human-driven global warming. Melt from Greenland already accounts for 25% of global sea level rise, double the contribution of Antarctica, and its share is growing. Rising waters are already exacerbating storm surge and causing sunny-day flooding in cities worldwide. Even by conservative estimates, Greenland could contribute another quarter-meter of sea level rise by 2100—within the lifetime of children living today. All told, the ice sheet holds enough water to raise seas by 7 meters—and no one knows whether, or how fast, that water will be unleashed.

As befits its mythical name, the domain of the Norse god of the dead, Helheim is truly an arbiter of Greenland's fate. The glacier is one of the ice sheet's primary drains, sliding into the sea at 8 kilometers per year and accounting for 4% of the ice sheet's annual mass loss. Its towering front, as tall as the Statue of Liberty, measures 6 kilometers across. Sea ice shed from the glacier chokes the fjord for tens of kilometers. The glacier's terminus has behaved erratically over the past 15 years, first retreating by 5 kilometers from 2002 to 2005 and then advancing and stabilizing for nearly 10 years. Then, in 2014, a more severe retreat began, sending the terminus 2 kilometers beyond its previous low. Meanwhile, the glacier has thinned by more than 100 meters, leaving a telltale "bathtub ring" high on the rock around the fjord.

Some two-thirds of Greenland's ice loss comes not as meltwater, but as chunks of ice that detach, or calve, from its 300 outlet glaciers—fast-moving rivers of ice that end in long fjords. Those narrow channels, hun-



PHOTOS: (LEFT TO RIGHT) DAVE SUTHERLAND/UNIVERSITY OF OREGON; P. VOOSSEN/SCIENCE



Researchers on the *Adolf Jensen* (left) probed Sermilik Fjord, one of the Greenland Ice Sheet's largest outlets, for warm Atlantic Ocean water infiltrating and eroding the ice.

dreds of meters deep, “are the bottlenecks,” Straneo says. They also are a fateful meeting place, where a glacier’s calving front encounters currents of increasingly warm ocean water. “These tiny systems are the connection between the ice sheet and the ocean,” she says.

Straneo’s past work showed that warm Atlantic water is penetrating Sermilik Fjord, which researchers once thought was dominated by Arctic waters. Here, it meets cold meltwater draining through channels beneath the ice. Straneo believes the emerging freshwater, buoyant because of its low salinity, mixes with the warm water and forms a plume that wells up against the glacier’s front, causing more melting and fracturing. It’s like the ice in your glass of whiskey, she says. “If you just put it in and don’t stir, it lasts a long time. If you stir it, it melts really quickly.”

To test the idea, she and colleagues need to go further, breaching Helheim’s protective veil of sea ice to observe the warm

plume directly. They also must track the behavior of the glacier itself in exacting detail.

The effort began this year: a 4-year, \$6 million project financed by the Heising-Simons Foundation in Los Altos, California. The project, modest in cost by Arctic standards, is attacking Helheim from every angle: drilling into firn—snow that has not yet compacted into ice—to gauge how much mass southeastern Greenland’s famously severe storms add to Helheim. Placing seismo-meters at the glacier’s terminus to sense the spread of hidden fractures. Recording the glacier’s calving four times a day with a pair of autonomous lasers, dubbed Atlas. Air-dropping hardened drifters that can capture conditions near the glacier front while surviving encounters with icebergs. Placing moorings up and down the fjord to probe its depths. And knitting all those observations together with advanced models of glacial fracturing.

Beyond Helheim, what Straneo and her peers find could feed into something grander: an international long-term ob-

serving system to monitor and assess the risks posed by the melting of Greenland’s most prominent glaciers. That endeavor could dispel much of the uncertainty about Greenland’s role in future sea level rise.

On arriving in Greenland, the team had a reminder of the urgency. A heat wave in Europe was forecast to reach them soon, causing melt unseen since 2012. And Straneo had heard that something phenomenal was happening up at Helheim’s front. A surge of meltwater was pouring out at the bedrock, pushing open a 500-meter-wide half-moon of water in the shadow of the glacier’s towering face. The effect had created a direct route to sampling the waters below. Such upwellings typically reach the surface for just a day or two. This plume’s size suggested it might last weeks.

STRANEO STARTED HER LIFE far from ice. Like many smart students in Italy, she was directed into physics. But she loved to sail and found herself drawn to the ocean and its

Northern watch

The Greenland Ice Sheet, which holds enough water to raise the seas by 7 meters, loses much of its ice through 300 glaciers, which flow into long, narrow fjords and break up into icebergs. But scientists have struggled to understand how human-driven warming drives such fracturing, making predictions of future sea level uncertain. At Helheim Glacier and its fjord, Sermilik, scientists have begun a campaign to track every part of the melting system to find patterns relevant across all of Greenland.

Graphic by **Chris Bickel**



Atlantic invasion

Frigid Arctic water was once thought to fill Sermilik Fjord. Moored instruments have now shown that a deep layer of warm water from the Atlantic Ocean is infiltrating all the way to Helheim's face.

Dwindling giant

Helheim is one of Greenland's largest outlet glaciers, draining 4% of its ice. It has retreated 7 kilometers (km) since 2002, thinning by more than 100 meters (m).

An opening

This summer, an unusual upwelling at Helheim's face created an ice-free pool that lasted weeks, allowing researchers to probe waters next to the glacier.

Turbulent mix

At the base of the glacier, a channel of meltwater flushes into the fjord. This meltwater, naturally light, drags warm Atlantic water up along the ice face.

Glacial flow

6 km

670 m

South laser scanner

Subglacial meltwater

Iceberg

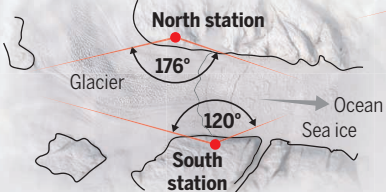
600 m

Arctic water

Warm Atlantic water

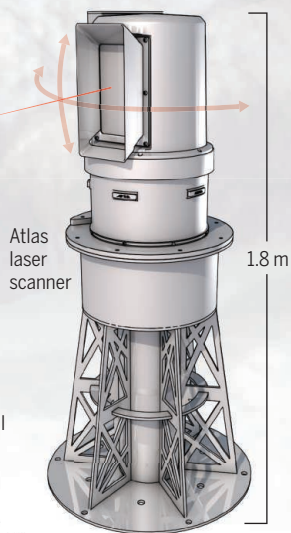
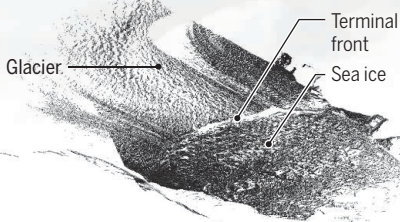
Laser sentinels

Atlas, two state-of-the-art autonomous laser scanners, survey Helheim and its sea ice four times a day during the melt season.



One billion points

Each run of Atlas generates some 40 million data points, a deluge of data.



large-scale currents, first as a modeler and then as a field scientist. She spent much of her academic life at the Woods Hole Oceanographic Institution in Massachusetts, and it was there, some 15 years ago, that she met Gordon Hamilton, a glaciologist at the University of Maine in Orono.

At the time, Hamilton and his student, Leigh Stearns, had begun to place GPS units on Helheim to track its startlingly rapid retreat. Helheim was a scientific backwater. It does not have the easy logistics that the National Science Foundation provides for researchers working on Greenland's western coast, and frequent North Atlantic storms blanket it in snow, making it harder to study than the glittering flat ice of the west.

Straneo had been focused on ocean currents, such as the "conveyor belt" that influences climate around the North Atlantic. But Hamilton convinced her that the smaller-scale currents in fjords could be key to an equally important process: Greenland's ice loss. In 2008, she and colleagues, including Hamilton, spent a season in Sermilik Fjord. They hired a small boat and dropped temperature and salinity sensors into the fjord on a fishing line, anchoring their moorings with cinder blocks.

Those first casts clearly showed something unexpected was going on. Scientists once thought the fjord would be filled with water that flows down from the Arctic, hugging Greenland's coast. But the measurements pointed to two layers of warm water in its depths: a deeper layer apparently heading up toward Helheim and a shallower one, cooler and less salty, heading back to sea. The deep flow was likely Atlantic water, ushered in by summer winds. The shallower flow seemed to originate as the warm water interacted with the ice, losing heat and salt in the process.

Year after year, Straneo returned to the fjord. Meanwhile, Hamilton's frustration with the limited data from the GPS units grew. Then, he met David Finnegan, a remote sensing scientist at the U.S. Army's Cold Regions Research and Engineering Laboratory (CRREL) in Hanover, New Hampshire, who uses reflected laser light to map terrain. What if they used such a laser to constantly monitor Helheim's front? Tying together such fracturing with the influx of Atlantic water could help them figure out what role, if any, the water plays in the loss of ice.

In 2012, Finnegan's team perched a prototype autonomous laser scanner on one of the fjord's walls, overlooking Helheim's terminus. The scanner played a beam of infrared light across the glacier, its front, and the sea ice, tracking the calving process iceberg by iceberg. The data dropped jaws when

Finnegan presented them at a meeting in 2013. This was big data come to glaciology, each reflected point tied to coordinates in space—as though the team suddenly had one million GPS units on the glacier at once.

Even with those two complementary lines of evidence, Helheim's ice loss defied explanation. "Each year was kind of different," Stearns says. Figure out a mechanism for 1 month, and a year later it was irrelevant. Too many variables, such as the flow of meltwater under the glacier and the temperatures beneath the sea ice, could not be tied down. Only a sustained monitoring system, targeting every element of the glacier and fjord, could show how the system delivered ice from the glacier to the ocean and how a warming climate might influence it.

That vision took a step toward reality just after Finnegan's talk, when Cyndi Atherton approached him. Atherton, who had joined Heising-Simons to direct its scientific grants, wanted to fund research that could make a room gasp. "What would you do with a blank check?" she asked. Heising-Simons began to finance the development

"These tiny systems are the connection between the ice sheet and the ocean."

Fiamma Straneo, Scripps Institution of Oceanography

of the Atlas scanners, which could capture the entirety of Helheim's crumbling tongue from perches on both walls of the fjord.

Three years into the work, tragedy struck: Hamilton died in 2016, at 50 years old, after his snowmobile fell 30 meters into an Antarctic crevasse. The researchers left behind hardened their desire to understand Helheim. Last year, Heising-Simons asked Straneo and Stearns to assemble a team that would tie down all Helheim's variables at once. The project would be a proof of principle, Atherton said, "that will allow other governments to say this is valuable, and we're going to extend from this site to other sites."

The work began this summer.

THE 39-METER JENSEN, a former Danish fisheries research ship and icebreaker, is far removed from large modern oceanographic ships, where scientists rarely get to touch an instrument. Its crew of five Greenlanders keeps it running with a mix of grease, tobacco, and gumption. The scientists, too, have to improvise around limitations such as the *Jensen's* 1960s vintage winch. When the team retrieves the carousel, a gray cylindrical frame outfitted with sensors and large sampling bottles, the winch is too feeble to hoist it on deck. That means an awkward transfer to the ship's crane.

It's just how the modest Straneo—who has had the same blue fleece since she was 18—likes it. "We still do oceanography the old-fashioned way," she says. "You kind of have to do everything yourself."

The *Jensen* had taken a back route from the port of Tasilaq in southeastern Greenland, threading through narrow channels before sailing out into Sermilik Fjord. Peaks towered on both shores. A thin fog filtered cathedral light onto glass-still water. To the west, the ice sheet bulked vast and gray behind the peaks. Icebergs, most calved by Helheim, were constant companions—some white, some sparkling blue, some sooty black—often dwarfing the *Jensen* as it steered around them like a tugboat around an oil tanker. Every so often, the quiet was broken by the waterfall rush of violence as icebergs flipped over, agitated by the ship's wake.

Beyond collecting and placing long-term moorings, the team wanted to measure the salinity and temperature of the water column within the fjord, as far north as the ice would allow. The work has its hazards:

One year, the ice was so thick on the way to retrieve a mooring that the captain had a panic attack and turned back. A few days later, an iceberg destroyed that mooring. But Straneo was confident that the old icebreaker

was up to the task.

After she could not retrieve the first mooring, three others came back with little drama, their spherical red-and-white floats popping up near the ship. Each mooring monitored a different depth to capture the Arctic-fed water near the surface and the Atlantic layers beneath it. "It looks so easy when they come up," Straneo said. "And it's so painful when they don't."

After several days in Sermilik's mid-section, the *Jensen* steamed closer to Helheim. The icebergs and their detritus grew denser, until the fjord seemed more ice than water. A mate climbed to the crow's nest to spot hazards, and the *Jensen* began to plow through human-size bergs to avoid their larger cousins. Finally, the ice defeated the crew. Helheim's cliff face, some 25 kilometers away, loomed into view for a few minutes before a fairy-castle iceberg obscured it.

The approach was close for Straneo, but it still left a large gap in her data. This year, other opportunities to fill it would come along. Her collaborators would launch a long-lived drifter that can navigate beneath sea ice. And, after the cruise, Straneo's team planned to take a helicopter out to the open pool and drop sensors straight into the plume of meltwater.

EVEN AS THE *JENSEN* navigated the fjord, two teams monitored the glacier itself. Near its front, Finnegan and the CRREL scientists tended to the autonomous Atlas lasers, one mounted on each side of the fjord. The researchers had also agreed to help Sridhar Anandakrishnan, a glacial seismologist at Pennsylvania State University in State College, deploy 15 small seismometers, called geopebbles, on the glacier just behind the calving front—a feat possible only from the air.

After a career working in Antarctica, it was Anandakrishnan's first experiment in Greenland. In the helicopter, his eyes widened on seeing the glacier. It was nothing like the flat, solid marine glaciers in Antarctica. Helheim was gouged with crevasses some 20 to 30 meters deep; its southern flank was such a crumpled mess that it was hard to tell where the glacier stopped and sea ice began. "It's so spectacular," he said. "It's so scary."

Over and over that day, the helicopter pilot descended to within centimeters of the cracked ice. Two tethered CRREL researchers hopped out. They drilled a hole, dropped in a geopebble handed to them by Anandakrishnan, and marked it with an orange flag. The devices would capture the seismic signals that shudder through the ice as it fractures—data that Anandakrishnan hoped to assemble into a 3D image of the under-ice channels that carry meltwater from its surface to the sea. The water "collects just like any river into larger and larger streams," he says, "until you have the Nile running down the bottom of Helheim."

First, Anandakrishnan needed to see whether his geopebbles worked. They were designed to transmit wirelessly so that a hovering drone could harvest their data. Two days after inserting the instruments, he and Marc Volpe, a gangly aerospace engineering student, took their homegrown drone out for a test flight. It looked the part: Two plastic pipes with a rotor on each end were strapped to a central metal axis about as long as a hockey stick. The landing gear was made of plastic foam.

The first few short test flights, launched from as close to the glacier as they could safely go, ended in a crash, or what Volpe called "an unintended rapid reconfiguration." The drone's simplicity meant it could be rebuilt, and by late morning of their second day, they were ready to send it out over the ice. "All clear," Anandakrishnan said. "No children or polar bears." But after

skimming more than 1 kilometer over Helheim, the drone began to spin like a drunk, and they nearly lost it before the radio signal returned and they called it back.

Finally, the day waning, Anandakrishnan asked Volpe whether he wanted to try again. "It's not your drone, it's not your money," Anandakrishnan said. "Want to go for it?"

"Yeah, I think so," Volpe said.

The drone again started to fly sideways—but in the right direction. One kilometer passed. Two. "Come on!" Anandakrishnan yelled. Three kilometers. "Holy crap, it's there!" Volpe said. The drone hovered for a minute over the seismometer and then darted back toward home. "Did it connect to the pebble?" Volpe asked. Not only did it connect, Anandakrishnan said with glee, but "we got a big chunk of data."



Fiamma Straneo has spent the past decade probing the fjord's currents. This summer, open water near the glacier gave her a rare chance to sample there.

HOUSED IN A CONICAL GRAY TURRET on a fjord wall, an unending mass of rock and moss, an Atlas scanner watched the ice sheet decay. Below the laser scanner lay the camp that has been the CRREL team's summer home for the past half-decade. A nearby waterfall supplies drinking water, no filtration needed. A slanted rock slab offers dinnertime views of the sun setting over Helheim.

The team there felt Hamilton's absence keenly. "Gordon would have never allowed this," Finnegan said as he poured hot water into plastic bags of freeze-dried food. "He believed in a good meal after a hard, long day of fieldwork." The void was even more pronounced this year: Stearns, Hamilton's former student and successor, was on maternity leave.

One morning, the CRREL team clambered up a cliff to Atlas. CRREL geoscientist Adam LeWinter gave it a friendly twist to say hello. It whirled back to its

rest position, gears growling at the interruption. The researchers cleaned, greased, and calibrated it while downloading data. They replaced a weather station and updated the batteries. Two reinforced vertical solar panels bracketed the device; several weather-protected black cubes contained methanol fuel cells for the long Arctic night. "The only fuel that doesn't freeze," Finnegan said. "But the exhaust does, which is problematic."

The dream of running Atlas year-round had proved elusive. During the summer, the two scanners work flawlessly, whirring to life every 6 hours. But each winter, they've found new ways to die. The team took bets on when the lasers would fail this winter. Ananda Fowler, a remote sensing scientist at CRREL, raised eyebrows with his forecast: They'd survive. "It feels good, man," he said. "I think this is the year."

The billions of data points, and counting, that the scanners have collected have proved hard to interpret. But the continuous measurements have begun to reveal, for example, the extent to which sea ice dropped by Helheim inhibits later calving and how crevasses open and spread. This summer, the lasers were trained on the ice around the open pool, tracking floating ice to gauge the upwelling plume's strength and watching to see whether the ice cliff was decaying faster than usual. In the past few years, the glacier has retreated so much that the scientists had to shift Atlas's field of view by 15° so that it could still observe the receding ice face.

Helheim was more active than the team could remember, cracking and grumbling. At the camp, heads popped up like prairie dogs each time a rumble and a whoosh signaled another collapse of ice into the open pool some 3 kilometers away. Within minutes, the force of the upwelling cleared the pool and stasis returned.

One day, a red helicopter clattered into view. The *Jensen* had docked at Tasilaq the previous day; now, Straneo and others were collecting data from parts of the fjord the ship could not reach. They hovered near the ice, a mosquito against the white wall, getting a close view of the pool. Before they could sample, Helheim began to grumble, and the pilot shot away, landing at a safe distance on the sea ice. "I can't believe we set down on that berg," Jamie Holte, an oceanographer in Straneo's lab, said later. They ultimately flew back to the pool, and Helheim remained docile enough for Straneo



Helheim Glacier has retreated 7 kilometers since 2002 while thinning dramatically, leaving ice-choked waters and a telltale “bathtub ring” on the fjord’s walls.

to shoot a disposable probe into the water to measure temperature and salinity.

Afterward, the helicopter landed at the camp. The upwelling was astonishing, Straneo reported: “The warm water in the plume is all the way up to the surface.” Later, data collected by a drifter showed that, for weeks, warm Atlantic waters had flowed 100 kilometers up the fjord and then welled up against Helheim’s face. “It confirms this upwelling is driven by the glacier,” Straneo said later, and appears to be playing a role in Helheim’s demise.

“Ten years of work and this is what you get,” she added, staring at a record of water temperature and salinity on her laptop, her voice a mix of pride and bemusement. The victory was hard-won after a disappointing end to their cruise. A second mooring, off the coast, hadn’t resurfaced. And another attempt to recover the first mooring had failed.

A CLEARER PICTURE of Helheim’s dynamics, and how they relate to larger oceanic trends, is beginning to emerge from all the trial and error. For example, Straneo says that this summer, the data suggest conditions were unusual compared with those in recent years. Water temperatures in the fjord were “quite a bit warmer”—some 0.2°C above their previous high. “The moorings agree that we had a ramping up.” A slightly warmer North Atlantic, it appeared, had

conspired with the robust surface melting to intensify the warm plume at Helheim’s face, potentially increasing calving.

The researchers have found that, in general, the warmth of the fjord’s deep water tracks larger North Atlantic temperature swings. That finding suggests ocean measurements could help serve as a rough predictor of glacier retreat. Straneo and Donald Slater, a glaciologist and postdoc in her lab, have already used a model based on that connection to make a prediction: By 2100, one-quarter of Greenland’s glaciers will retreat more than 10 kilometers, if emissions and ocean warming continue unchecked.

But, “We’re still far from being able to make reliable projections,” says Mathieu Morlighem, an ice sheet modeler at the University of California, Irvine, who is also working on the Helheim project. “If we don’t get ice dynamics right at the coast, there’s no way we get a decent projection.”

Accomplishing that will take further scrutiny of Helheim. The lasers and replenished moorings will remain in place at least until 2022. Next year, the team will return with radar-equipped drones to map the topography beneath Helheim, looking for hidden slopes that could slow its retreat. Farther inland, they will continue to study trends in snowfall and melt by using satellite measurements and core samples of compacted snow.

The team will turn to computer modeling to unify all their observations: the seismic wiggles, the currents, the calving, the snowfall and ponding meltwater. Douglas Brinkerhoff, an ice sheet modeler at the University of Montana in Missoula, will write the code that pulls those observations together, adapting techniques from weather modeling to suck in data. Meanwhile, Morlighem will develop a high-resolution model to replicate just how multiple processes conspire to fracture the ice and erode the glacier’s face.

“What I told Mathieu is, at the end, you need to be able to reproduce what Helheim has done,” Straneo says. “And if we can explain this glacier, we should be able to scale up our understanding of the physics to other systems.”

AFTER 3 DAYS with one Atlas, Finnegan’s team moved to the opposite side of the fjord, to ready the second scanner for the winter. Then, a few days later, the scientists were gone, their instruments left behind. Meanwhile, Greenland continued to crumble, losing 329 billion tons of ice this year—a near record.

For now, Helheim endures. The Atlas lasers are keeping watch as the days shorten, whirring and dancing their invisible lights across the ice. And in the fjord, a lost mooring, holding fast to the murky bottom, records the signal of an ever-warming sea. ■

SEEING FOSSILS IN A NEW LIGHT

Protein residues
reveal physiology
and family ties from
the age of dinosaurs

By **Gretchen Vogel**

Photography by **Lindsey Lege**



In the bowels of Yale University's Peabody Museum of Natural History, Jasmina Wiemann yanks open a drawer in a floor-to-ceiling specimen cabinet. She lifts out a wickedly sharp, sickle-shaped dinosaur claw, black as coal. "This is the type specimen of *Deinonychus*—the basis for the *Velociraptor* in the *Jurassic Park* movies," she says. The black color signals something just as striking. The fossil isn't just a mineral replica of the original claw. It is likely two-thirds dinosaur residue, Wiemann says. "I bet this specimen is maybe 70% organic material by volume—more than we'd think!"

That fossils can harbor organic matter isn't new. Whole fields of science have sprung up to decipher ancient DNA and intact proteins. But most researchers think that in fossils as old as the *Deinonychus* claw, most of the useful sequences of those molecules have long vanished. Now, Wiemann and her Ph.D. adviser, Yale's Derek Briggs, have devised a way to extract information locked in degraded proteins, even in fossils hundreds of millions of years old. "This [kind of] molecular preservation is really common, and we just didn't know," Wiemann says.

She and Briggs have shown how, when conditions are right in the weeks and months after an animal dies, cellular proteins can react with lipids and sugars. The process transforms the proteins into a mix

of hardy polymers that repel water, resist microbes, and are impervious to heat. The polymers are chemically similar to those formed when meat browns or toast burns—and they can apparently last for eons.

Other researchers have claimed to find intact proteins more than 100 million years ago, in the age of dinosaurs (*Science*, 15 September 2017, p. 1088), but the field has remained skeptical of such ancient preservation. No one could explain how proteins managed to survive the degradations of time, says paleontologist Maria McNamara of University College Cork in Ireland. Wiemann "very nicely came up with this very, very clever mechanism" for how proteins could persist after all, in an altered form.

After a proof-of-principle paper last year, Wiemann and Briggs are applying their nondestructive technique—shining a laser on specimens to reveal ancient chemical bonds—to help solve paleontological mysteries. This week at a meeting in Australia, they planned to report how they used protein residue data to help resolve where turtles fit on the vertebrate family tree, and to support the idea that pterosaurs, the largest animals ever to fly, were warm-blooded.

The technique is new, so "it needs to be validated by more fossil and experimental work," McNamara says. And the method by itself can't resolve evolutionary puzzles with certainty. But she and others find the

The laser of a Raman spectrometer shines on a *Tyrannosaurus rex* tooth, gathering data about the organic material still locked inside it.

chemical mechanism convincing. "It's a completely new level of understanding of preservation. They are shedding light on the why and how," says Jingmai O'Connor, a paleontologist at the Institute of Vertebrate Paleontology and Paleoanthropology in Beijing. "It's incredible how [Wiemann] is revolutionizing our field, opening so many new doors by applying chemistry to a field where chemistry has rarely been applied."

FOR WIEMANN, the first clues to how biomolecules might persist for hundreds of millions of years came from dinosaur eggs. As an undergraduate and master's student, she worked with a team led by Martin Sander at the University of Bonn in Germany that showed that 67-million-year-old dinosaur eggs were blue-green, not white. To isolate the color pigments, Wiemann dissolved pieces of fossil eggshell in a solution that removed the calcium. Sometimes she found pliable brown residues at the bottom of her test tubes. Under the microscope, the residues resembled the organic matrix of eggshells, and she wondered whether they were bits of original tissue. "It was quite exciting to see," she says. But she didn't have time to figure out exactly what she was seeing.

For her Ph.D., she went to Yale to join Briggs's lab, where she set out to identify those brown dregs. She found more residues when she decalcified pieces of fossil bone and teeth. "The residue you get is very different from fresh proteins," she says, but under the microscope it showed tantalizing hints of soft-tissue structures—blood vessels, cells, even nerve projections.

Wiemann and Briggs—an expert in soft-tissue preservation—noticed that residues were most likely to come from fossils of a specific type: black or brown ones from lighter-colored rocks that formed in shallow seabeds or iron-rich sandstones. Those environments are oxidative, rich in reactive oxygen molecules and dissolved metal ions, and the water that enters decaying bones and tissues is alkaline—conditions that promote biochemical reactions called glycooxidation and lipoxidation. Food chemists know them well. Called Maillard reactions, they occur anytime something is toasted, grilled, caramelized, or "browned," turning proteins, fats, and sugars into tough, complex polymers that are brown and often taste delicious.

As a teenager, Wiemann had taken university level classes in organic chemistry. "So I knew about the Maillard reactions and how you could go from a protein to a water-resistant thing," she says. She wondered whether something similar had happened to the proteins in the fossilized tissues.

To find out, Wiemann turned to Raman spectroscopy. Many biochemical techniques search for a specific compound, Wiemann says, "but Raman spectroscopy is more exploratory." It uses laser light to identify the types of chemical bonds in a sample. Different bonds absorb different wavelengths, leaving a fingerprint in the spectrum of the reflected light. The technique enabled Wiemann and Briggs to confirm that the brownish residues were indeed made of complex polymers, the end products of glycooxidation and lipoxidation. When the researchers artificially fossilized modern bones and eggshells, heating them under oxidative conditions in the lab, the modern tissues formed the same kinds of compounds, the team reported in *Nature Communications* in November 2018.

Different proteins form different poly-

mers, so despite their transformation, ancient molecules retain some of their original chemistry (see graphic, below). The result is a new kind of molecular tool for studying ancient life—one that complements ancient DNA and proteins, Briggs says.

Although ancient DNA carries the most detailed biological information, it degrades most quickly. Relatively intact proteins can persist for nearly 4 million years and can still distinguish between closely related species. With protein residues, "the information is again reduced," Wiemann says. The polymers don't preserve 3D structure or a complete sequence of amino acids. But the compounds are incredibly stable, preserved

tebrate fossils," he says. Wiemann spent her evenings raiding the tall cabinets, seeking fossils with the telltale dark color in light sediment. "No one ever comes here in the evenings," she says of the collection rooms. "It's actually fun."

The scans don't damage the specimens, so curators agreed to lend them for study. Wiemann's office, down the hall, is littered with specimens from many geologic periods and across the tree of life, each in its carefully labeled box. "Curators definitely appreciate that it's nondestructive," she says.

She has so far analyzed more than 100 specimens with the Raman spectrometer, which looks like a large microscope.

She examined an *Allosaurus* specimen from Carbon county in Utah, so black it glistens; fish from the famed Green River deposit in Wyoming; the claw from *Deinonychus*, a species that inspired the first suggestions that some dinosaurs could run quickly and were the ancestors of birds; and her old favorites, fossil eggshells.

With this still-growing database, she first tested whether the spectral signatures could reveal relationships among ancient animals. By comparing slight differences in the number and height of spectral peaks, a computer could correctly slot eggshell samples from more than a dozen dinosaurs, including early birds, onto a phylo-

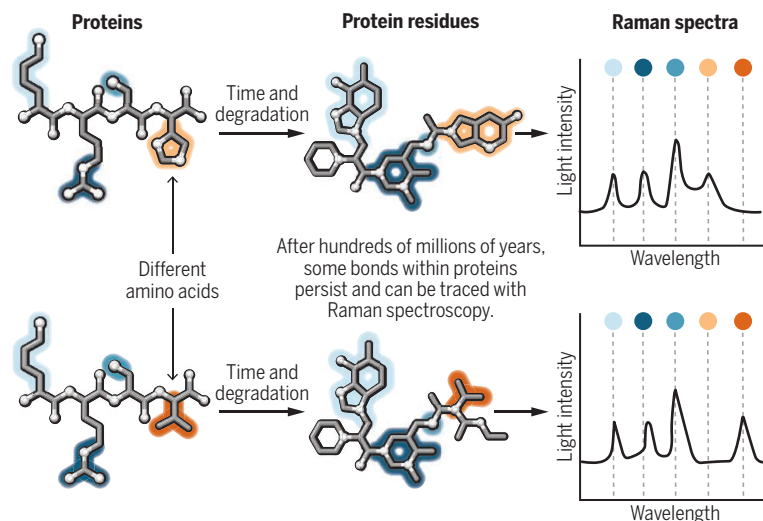
genetic tree. Samples of bones and teeth matched about 60% of known relationships, Wiemann planned to announce at the Society of Vertebrate Paleontology meeting in Brisbane, Australia, this week.

"This is not something that is going to tell you how 10 hadrosaurs you found in a quarry were related, but if you find bits and pieces of turtle, stem bird, and crocodile, it can probably help you tell them apart," she says.

In another talk at this week's meeting, Wiemann's and Briggs's colleague Dalton Meyer planned to describe how they compared Raman spectra from bones of many extinct reptiles to find where turtles might belong on the reptile tree—a persistent puzzle. The spectra suggest turtles' ancestors, which lived more than 200 million years ago, were more closely related to dinosaurs (and modern birds) than to the ancestors of crocodiles, snakes, and lizards.

How ancient bonds can cheat time

In certain environments, a dead organism's proteins degrade into hardy polymers. Different proteins form different polymers, so Raman spectroscopy, which can distinguish various kinds of bonds, can offer clues to proteins' original chemistry, even after eons.



"through deep time," Briggs says. Wiemann says they have identified protein residues in 500-million-year-old fossils from Canada's Burgess Shale in British Columbia.

That claim is startling, but "the chemistry makes sense," says Evan Saitta, a paleontologist at the Field Museum in Chicago, Illinois. "If you have ever scrubbed a grill after cooking, you will know that these protein products are stable at high temperatures—and are clearly insoluble."

To understand the meaning of the spectra, though, Wiemann had to build a database with the spectral signatures of many fossils and modern samples. And she had to find out whether those spectra could tell her anything interesting about ancient life.

LUCKILY, WIEMANN WORKS at a museum with a "colossal" collection, Briggs says. The Peabody Museum holds "100,000 vertebrate fossils and about 4.5 million inver-



Derek Briggs (left) and Jasmina Wiemann sought specimens still rich in protein residues in Yale University's Peabody Museum, looking for dark fossils in pale sediment.

Wiemann, Briggs, and colleagues have applied the technique to organic matter from even further back in time. *Tullimonstrum*, also known as the Tully Monster, is a mysterious creature from the Mazon Creek fossil beds in Illinois. It lived more than 300 million years ago and has stumped paleontologists for decades. A soft-bodied oval with a long appendage, it has sometimes been identified as a vertebrate, some sort of worm, or a strange swimming snail. But Raman spectra of the animal's teeth suggest they were made of keratin or collagen, proteins made only by vertebrates and their relatives, Victoria McCoy, a graduate of Briggs's lab now at the University of Wisconsin in Milwaukee, planned to report at the meeting. That finding suggests the creature was some kind of vertebrate, rather than a snail or worm.

McCoy, Briggs, and their colleagues had already reached a similar conclusion from analyzing the shape of more than 1200 Tully Monster specimens, which they published in 2016 in *Nature*. The protein residue analysis "is helping to confirm our original morphology conclusions with chemical data, which is less ambiguous—and quite exciting," Briggs says.

Wiemann also told the meeting how protein residues may point to whether an extinct animal was warm-blooded. The reactions a cell uses to produce energy also generate side products called free radicals,

which can trigger Maillard-like reactions and produce polymers similar to those in the fossil protein residues. The cells of warm-blooded animals with faster metabolisms carry out more of those reactions than do cold-blooded ones. Wiemann realized that if she could correct for the Maillard reactions induced by fossilization, she might detect variations due to metabolic differences. She tracked fossilization reactions in her experiments with modern samples and concluded that the ratio of two key types of chemical bonds in a fossil's Raman spectrum might point to metabolic rate.

She tested the idea, and at the meeting she planned to report that her results fit with what's known or suspected about metabolism in fossil and living creatures. Mammals, pterosaurs, and two-legged dinosaurs such as the fast-moving *Allosaurus* and *Deinonychus* had the signature of warm-blooded animals. Mammallike reptiles that lived 300 million years ago turned up as barely warm-blooded, and quadripedal dinosaurs such as *Triceratops* appeared to have even slower metabolisms. The ancestors of lizards and snakes appeared to be cold-blooded.

"We've only been able to guess about metabolic rates using features like bone histology or inferred brain size," O'Connor says. The new method may offer a more direct route to metabolism. She suspects warm-bloodedness evolved several times in the

ancient bird lineages she studies and says her lab will soon start a project using Raman spectra to explore metabolic rates in bird fossils.

"That these [residues] can tell us something about basal metabolic rate is sort of mind-blowing," says Lawrence Witmer, a paleontologist at Ohio University in Athens. "It takes a conceptual leap, really an outstanding creativity, to see these connections we haven't seen."

Some researchers caution that independent labs haven't yet replicated the work. And they say Wiemann hasn't definitively ruled out that some spectra might stem from contamination—for example, from bacteria that colonized the fossil. "You don't know whether you're measuring the original stuff, more modern stuff, or a mixture of both," says Johan Lindgren, a paleontologist at Lund University in Sweden.

Wiemann says bacterial residues and other contaminants have characteristic Raman signatures that she can rule out. But back among the Peabody's shelves, she agrees that plenty remains to be done. "We have to optimize these methods," she says. "That's not something one person can do alone. It's something that the whole field has to take on." ■

With reporting by Elizabeth Culotta in New Haven, Connecticut.

INSIGHTS

POLICY FORUM



CLIMATE POLICY

Double counting and the Paris Agreement rulebook

Poor emissions accounting could undermine carbon markets

Lambert Schneider¹, Maosheng Duan², Robert Stavins³, Kelley Kizzier⁴, Derik Broekhoff⁵, Frank Jotzo⁶, Harald Winkler⁷, Michael Lazarus⁵, Andrew Howard⁸, Christina Hood⁹

The 24th international climate conference in Katowice, Poland, in December 2018 was a major achievement in the multilateral response to climate change. More than 190 countries managed to agree on nearly all elements of

a comprehensive rulebook that puts flesh on the bones of the 2015 Paris Agreement. The rules require, for the first time, that all countries provide detailed information on their climate change mitigation targets and regularly report on their progress in implementing and achieving them. However, one important chapter is still missing: rules for international carbon markets discussed under Article 6 of the Paris Agreement. Competing views on how to avoid “double counting”—counting the same emission reduction more than once

to achieve climate mitigation targets—were a major roadblock to reaching consensus. Completing the missing chapter on Article 6 will be one of the key tasks when countries reconvene at the 25th international climate conference in Santiago, Chile, in December of this year. We highlight why resolving double counting is critical for achieving the goals of the Paris Agreement and identify essential ingredients for a robust outcome that ensures environmental effectiveness and facilitates cost-effective mitigation.

ILLUSTRATION: BENEDETTO CRISTOFANI/SALZMANART



Carbon markets can involve three distinct yet closely related levels of actions. First, national or regional jurisdictions can establish policies, such as emissions trading systems, that enable firms to trade emission permits, or credits for having reduced emissions relative to a baseline. Second, jurisdictions can link their policy instruments, which allows these permits or credits to be traded across international borders (1). Third, and our focus, Article 6 of the Paris Agreement establishes a framework that allows countries to count these international transfers when demonstrating achievement of their targets under the Paris Agreement.

Carbon markets provide flexibility in where and when greenhouse gas (GHG) emissions are reduced and thereby can lower the aggregate cost of achieving climate mitigation targets. This could help

governments adopt more ambitious targets (1–3). Efficiency gains associated with carbon markets could thus help achieve the deep emissions cuts that are necessary to reach the goal of the Paris Agreement of holding the increase in the global average temperature to well below 2°C above pre-industrial levels and pursuing efforts to limit it 1.5°C. If not robustly designed and implemented, however, carbon markets could lead to greater emissions and higher costs and thus undermine the agreement (4).

AVOIDING DOUBLE COUNTING

Double counting of emission reductions is one of the main ways in which the integrity of carbon markets could be undermined. If it is not prevented, actual GHG emissions could end up being greater than the aggregated achievement that the countries (or private sector entities) participating in the carbon market report (5, 6). Avoiding double counting is thus fundamental for the integrity and healthy functioning of any carbon market and critical for the credibility of the Paris regime.

In the context of the Paris Agreement, a robust system to account for international transfers of emission reductions is the main ingredient needed to avoid double counting. The basic principle is simple: The international transfer of emission reductions should not lead to higher total emissions than if the participating countries or entities had met their targets individually (5, 6).

Article 6.2 of the Paris Agreement establishes such an accounting framework. It avoids double counting through a form of double-entry bookkeeping, referred to as “corresponding adjustments.” As with bank transfers, an entry in one account requires a corresponding, opposite entry to another account. Under the Paris Agreement, the relevant currency is emission reductions: The country selling emission reductions makes an addition to its emission level, and the country acquiring the emission reductions makes a subtraction. Both countries prepare an emissions balance in which the country’s target level is compared with its emissions, adjusted for any international transfers of emission reductions (7).

To implement this approach, negotiators are considering various further ingredients—in particular, requirements for countries to clarify their targets in terms of GHG emissions; to track international transactions of emission reductions through electronic registry systems; and to regularly report on their emissions and carbon market transactions, subject to a technical review.

Addressing double counting is critical because nearly half of the Parties to the Paris Agreement have signaled their intent to use

carbon markets, many of them as sellers of emission reductions (8). The European Union and Switzerland, for example, agreed to link their emissions trading systems and to count the resulting transfers of emission reductions toward their targets under the Paris Agreement. A similar arrangement might be made if the United Kingdom leaves the European Union. Several countries have announced their intent to achieve net-zero emissions between 2030 and 2050, including through the purchase of emission reductions from other countries. Some countries, such as Japan and Switzerland, have already started purchasing emission reductions.

The largest demand for emission reductions may not come from a country but from airlines. Because of the difficulty of attributing emissions from international aviation to a particular country, the Kyoto Protocol mandated the International Civil Aviation Organization (ICAO) to address these emissions. In 2016, ICAO adopted a new global scheme that requires airlines to offset any increase in carbon emissions from international flights above 2020 levels. Over the scheme’s operational period from 2021 to 2035, airlines could demand as much as 1.6 billion to 3.7 billion emission reduction credits (9), compared with about 2 billion credits purchased by countries to meet their Kyoto commitments in the period from 2008 to 2020.

Next to using carbon markets for compliance purposes, organizations and individuals increasingly purchase emission reductions to voluntarily offset their emissions. There is considerable debate whether double counting needs to be avoided for such purchases or whether these emission reductions can be used by both countries to achieve the Paris targets and the organizations or individuals to offset their emissions (10).

If these transactions are to be credible, they must be underpinned by international accounting rules that prevent double counting. But why are such rules so highly controversial in international negotiations? Resolving double counting is politically challenging because countries have different interests and hence different interpretations of what the requirements of the Paris Agreement mean. It is also technically challenging because countries communicated rather diverse mitigation pledges under the Paris Agreement, which makes accounting complex.

POLITICAL OBSTACLES

The Paris Agreement is explicit that double counting shall be avoided. Still, countries wrangle not only over how double counting should be avoided but also what constitutes double counting and whether it should be avoided under all circumstances (11).

Some countries have proposed that seller

countries should not have to apply corresponding adjustments if the emission reductions are generated under the new, internationally governed crediting mechanism established by Article 6.4 of the Paris Agreement. This new mechanism is commonly viewed as a successor to the Clean Development Mechanism (CDM), which allows developed countries, who have mitigation commitments under the Kyoto Protocol, to acquire emission reduction credits generated from projects in developing countries, who do not have such commitments under Kyoto. The CDM and the new Paris mechanism both require that certified emission reductions must be “additional” (that they would not have occurred without the carbon market incentives). Brazil, supported by a few others, has argued that the requirement of additionality obviates the need for corresponding adjustments by seller countries because it ensures that the emission reductions go beyond the climate action that the country would pursue to achieve its Paris mitigation target. This position would implement accounting similar to the Kyoto Protocol, in which only developed countries have climate mitigation targets, so there would be no need for developing countries to account for transfers of emission reductions. However, it could result in double counting in the new context of the Paris Agreement, under which all countries have pledged climate mitigation contributions. Most countries therefore support that corresponding adjustments be applied by both selling and acquiring countries under the new Paris mechanism. Disagreement over this matter was central to the failure to reach consensus on carbon market rules in Katowice (12).

Countries are also wrestling with avoiding double counting across different United Nations regimes. Under ICAO, countries have formally agreed that double counting between countries’ mitigation targets and ICAO’s aviation scheme should be avoided (13). Yet under the Paris Agreement, some countries, most vocally Saudi Arabia, have taken the position that international rules under Article 6 should not address such double counting, arguing that Article 6 only refers to transfers of emission reductions to achieve Paris targets but not transfers to airlines, and that ICAO and the Paris Agreement are independent treaties. Without a requirement for countries to apply corresponding adjustments for emissions reductions sold to the aviation industry, however, there is a risk that these reductions are dou-

ble counted: once by the selling countries to achieve their Paris targets and once by airlines to achieve their obligations under ICAO. Failure to resolve this matter could undermine the integrity of ICAO’s scheme and cause some countries to abandon it.

Another controversy relates to how much international oversight is needed to ensure robust accounting (11, 12). To preserve integrity and avoid the risk of a race to the bottom, some countries, including Senegal and South Africa, argued for more international oversight—not only for the mechanism established by Article 6.4 but for any carbon market cooperation among countries. This could, for example, include more detailed rules rather than principles, or participation requirements that countries must satisfy to engage in transfers. Other countries—including Australia, Canada, Japan, and the United States—had opposed strong international oversight for bilateral carbon market approaches, arguing for more national sovereignty and flexibility in implementing bilateral carbon market cooperation. Countries are now moving toward an approach in which they report on how they ensure accounting in accordance with the Paris rulebook and their reports are subject to a technical review.

DIVERSE CLIMATE PLEDGES

Climate pledges made by countries under the Paris Agreement are diverse. Many countries have formulated their pledges as some form of GHG emissions targets, whereas others have used different metrics, such as targets for the penetration of renewable sources. Some pledges do not cover all sectors of the economy or all GHGs; some are conditional on the provision of support from other countries; and some have no quantitative targets whatsoever, only qualitative descriptions of actions or strategies. Countries have also chosen different time periods for their targets; many have pledged targets for a single year—most 2030, some 2025—whereas some have chosen a multiyear period, such as 2021 to 2030. And some pledges are simply unclear; for example, they lack a clearly defined scope of the target or express a target as a deviation from business as usual without having determined their business-as-usual emissions (14). All of these factors make accounting complex.

One important matter is how best to account for transfers in the context of different target time frames (5, 6, 11, 12). For example, South Korea has proposed that countries should be allowed to count emission reductions achieved in another country over many



years (for example, from 2021 to 2030) to achieve a target for a single year only (for example, 2030). This could undermine environmental integrity in various ways—for example, if the seller country would only account for transfers that occurred in its target year (for example, 2030) (5, 6). But how to account for transfers of emission reductions generated in pre-target years is an open question. Adopting multiyear targets or trajectories, although potentially politically difficult, would be much more tractable for carbon market accounting. It would ensure continuous accounting over time and provide for integrity and transparency.

There is also debate whether, and under which conditions, countries should be allowed to sell emission reductions from GHGs or economic sectors that they have not included in their targets under the Paris Agreement (for example, a country reducing and selling CH₄ emissions, whereas its Paris target only includes CO₂) (15). In this case, the transfer of these emission reductions would not lead to double counting, and corresponding adjustments would thus not be necessary on the part of seller countries. However, allowing such transfers without adjustments by seller countries could create a disincentive for them to include more sectors and GHGs in their future targets because doing so would compel them to make adjustments any time they wish to sell such emission reductions.

¹Oeko-Institut, Berlin, Germany. ²Institute of Energy, Environment and Economy, Tsinghua University, Beijing, China. ³John F. Kennedy School of Government, Harvard University, Cambridge, MA, USA. ⁴Environmental Defense Fund, New Orleans, LA, USA. ⁵Stockholm Environment Institute, Seattle, WA, USA. ⁶Crawford School of Public Policy, Australian National University, Canberra, Australia. ⁷Energy Research Centre, University of Cape Town, South Africa. ⁸Koru Climate, Bonn, Germany. ⁹Compass Climate, Paekakariki, New Zealand. Email: l.schneider@oeko.de; duanmsh@mail.tsinghua.edu.cn



To address this concern, some countries have proposed that international rules require that seller countries apply corresponding adjustments for all transfers, regardless of whether the emission reductions occur within or outside the scope of their Paris targets. This would create incentives for seller countries to expand the scope of their targets and make accounting simpler because it would avoid the need to determine whether emission reductions occur inside or outside the scope of their targets. However, such an approach could make it more difficult to use international carbon markets for reducing emissions that occur outside the scope of Paris targets. To resolve these issues, one option considered in the negotiations is a grace period for the application of corresponding adjustments.

TO SUCCEED IN SANTIAGO

Common international accounting rules for cooperation through carbon markets are essential, for two reasons. First, the credibility and integrity of the international climate regime could be undermined if countries would pursue their own, less robust accounting approaches. Second, countries or firms might refrain from using carbon markets if they do not have clarity regarding whether they can claim the emission reductions they acquire. Success in Santiago is therefore critical. We propose several principles to guide the negotiations.

First, a single set of common international accounting rules should apply under the Paris Agreement, irrespective of which

carbon market mechanism is used to generate emission reductions and irrespective of whether these reductions are used by countries to achieve their Paris targets or by other entities, such as airlines to achieve their mitigation obligations under ICAO. This is important for avoiding double counting and for creating a level playing field for international carbon markets.

Second, ensuring robust accounting, regardless of how mitigation targets are expressed, is essential. This is most easily achieved by accounting in common GHG emission metrics and over continuous multiyear periods, with corresponding adjustments applied to all relevant years, rather than only to single target years. For some countries, this could require clarifying what their current mitigation pledges mean in terms of GHG emission levels over time.

Third, the Paris Agreement foresees that over time, all countries will move toward economy-wide targets. Robust accounting would be greatly facilitated if all countries adopted targets that are economy-wide, cover all GHGs, apply to common multiyear time periods, and are expressed as GHG emissions.

Next to resolving double counting, negotiators will need to address other controversial matters to reach agreement in Santiago, including whether a proportion of carbon market transactions revenue levied to pay for climate change resilience should only apply to the mechanism under Article 6.4 or to all international transfers under the Paris Agreement; whether tradable emission units left over from the CDM or from overcompliance

Without agreed-upon rules, there is a risk that emission reductions are double counted—once by the selling countries to achieve their Paris targets and once by airlines to achieve their obligations under the ICAO. Failure to resolve this matter could undermine the integrity of ICAO's scheme and cause some countries to abandon it.

with the Kyoto Protocol targets may be used to achieve Paris targets after 2020; and how other environmental integrity risks, such as transfers that are not backed by actual emission reductions, should be addressed.

To build a solid basis for international cooperation that can cost-effectively combat climate change, the Paris Agreement needs international carbon market rules that ensure environmental integrity and avoid double counting. Otherwise, international carbon markets might instead seriously undermine this carefully constructed climate agreement. ■

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PERSPECTIVES

ECOLOGY

Societal burdens of nature loss

Interdisciplinary science and international policy collaborate to stem inequities

By **Patricia Balvanera**

The rapid decline of biodiversity predicts dire consequences for human society, according to the recent Global Assessment Report by the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (1). The report notes that up to a million species are threatened with extinction (2) and that many benefits humans obtain from nature have decreased over the last 50 years, a decline likely continue until at least 2050. If transformative changes are to be implemented, scientists and policy-makers must address questions about the deterioration of nature and the locations that bear the greatest resulting burdens. On page 255 of this issue, Chaplin-Kramer *et al.* (3) address these questions by presenting global models of the current status and future trends of three key contributions from nature.

Ecologists define nature as the diversity of living organisms and the interactions among themselves and with their environment, including plants, animals, water bodies, and landscapes. In the past two decades, researchers from the biophysical, social, and geographical sciences have developed a large body of research on nature's contributions to people (also called ecosystem services) (4, 5). Maps of nature's contributions to people are now being used to guide the design of policies and technological interventions (6, 7).

Nature can contribute to societal well-being in various ways (8). These can be material, such as natural resources that sustain human life (e.g., food from fisheries); non-material, such as nature's subjective impact on people's quality of life (e.g., inspiration); or regulating, such that living organisms and their interactions modulate environmental conditions. For example, water quality is regulated by organisms in the vicinity of lakes and rivers, such as plants and algae that remove and store potentially harmful pollutants; these include nitrates leached from upstream agricultural fields where nitrogen-

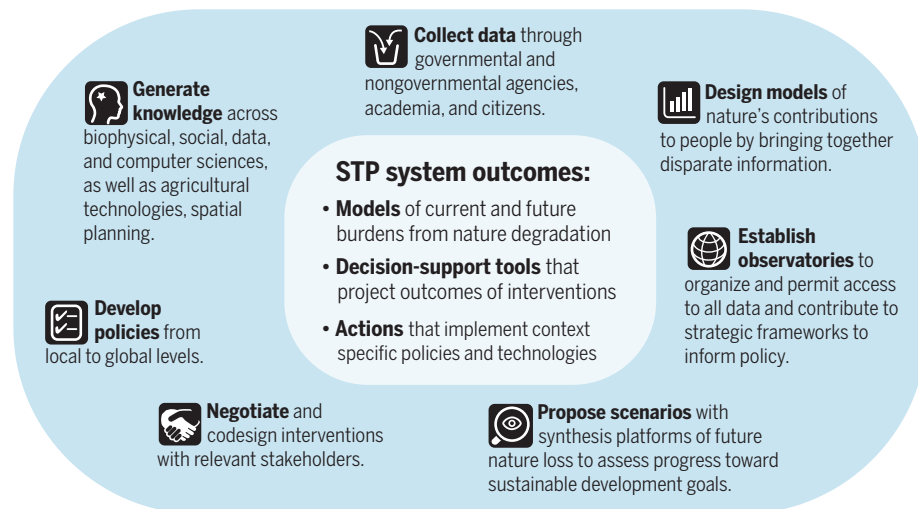
based fertilizers were applied to boost crop yields. Coral reefs, mangroves, seagrasses, and salt marshes attenuate the erosion and inundation impact of the ocean, which is driven by waves, wind, sea-level changes, and sea surges. Nesting sites of wild pollinators (e.g., bees) of fruits, seeds, and nuts contribute to the production of micronutrients. Chaplin-Kramer *et al.* focused their mapping on these three phenomena (all studied extensively in the past two decades) that are regulating contributions of nature to people.

nature's contributions: nutrient retention, coastal protection, and wild crop pollination. They also mapped the gap between the societal needs and nature's contributions, which likely results from nature degradation: pollutants not retained by vegetation before entering waterways, coastal hazards unmitigated by coastal ecosystems, and crop losses from insufficient wild pollination resulting from a loss of nesting sites. In addition, they overlaid their data with population data to identify which populations bear the largest threats to water, physical, and food security.

Over the past decade, powerful tools have been developed for mapping ecosystem services across space and time and modeling these data in alternative futures (4–7). Daunting challenges include bridging the rapidly evolving and disparate biophysical and societal data in ways that can be applied to the design of relevant policies and technologies (4–10). Chaplin-Kramer *et al.* provide maps of societal burdens stemming from the loss of nature at very high resolu-

A science, technology, and policy system to curb nature loss

Interdisciplinary scientists, policy-makers, and international alliances work together to map nature's degradation and its consequences and ultimately develop policies and interventions to address unequal burdens.



Most current state-of-the-art mapping approaches refer to a cascade that connects the attributes of nature; the potential and actual supply of a particular contribution to society; its flow or delivery to a particular area or user; and its societal demand, need, or value (4, 5). By contrast, Chaplin-Kramer *et al.* developed an innovative approach to assess where the contributions from nature are most needed and who bears the largest burdens from the deterioration of nature.

Chaplin-Kramer *et al.* mapped the societal needs from nature: total nitrogen runoff, total coastal risk, and total pollination-dependent crop production. Then, they mapped

tion, for every 300 m by 300 m bit of Earth. The maps for nutrient retention, coastal protection, and wild crop pollination present a worrisome picture: By 2050, 4 billion to 5 billion people will face water and food insecurity resulting from insufficient nitrogen retention and wild crop pollination, and hundreds of millions will lack coastal protection. It is alarming to imagine the magnitude of the predictions when researchers consider declines in other contributions of nature to people, such as a decrease in soil fertility or an increase in detrimental organisms.

Chaplin-Kramer *et al.* also pinpoint patterns of unequal societal burdens of nature

Instituto de Investigaciones en Ecosistemas y Sustentabilidad, Universidad Nacional Autónoma de México Apdo. Postal 27-3, Sta. Ma. De Guído, Morelia, Michoacán 58090, Mexico. Email: pbalvanera@iies.unam.mx

GRAPHIC: N. CARY/SCIENCE (MAPS) R. CHAPLIN-KRAMER ET AL. (3)

loss across the planet. Under all three future scenarios, Africa and South Asia will consistently harbor the most people enduring hardships of insufficient contributions from nature. A half-billion people in Africa, South Asia, and the Americas will face insufficient coastal protection. People who are most affected by the enlarged gap between societal needs and nature's contributions to people are those who are in direct contact with nature and do not have access to substitutes: for example, those who only drink water from streams with excess nitrogen, those who live along a barren coastline, and those unable to buy fruits, seeds, and nuts.

These results suggest the need to identify targeted interventions that restore nature or circumvent its degradation and to address the impacts of human population growth. For instance, agroecological technologies that increase crop diversity, reduce the need for nutrient supplementation, provide habitats for pollinators, and stabilize yields in the face of climate change (11) are most needed where nutrient retention and wild crop pollination gaps are highest. Coastal planning can prioritize conservation and restoration where the coastal protection gap is highest and harmonize infrastructure development accordingly (12). Enhanced international cooperation can include more countries willing to develop national strategies for protecting pollinators and their habitats [currently, 25 countries belong to an international coalition to promote pollinators (13)]. Transforming the visions and values of a handful of transnational corporations that shape most of the global material flows from nature (14), together with science-based policies and technology, can contribute substantially to forging sustainable futures. ■

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NEUROSCIENCE

Modulating anxiety and activity

A regulator of inhibitory neurotransmission is essential for benzodiazepine actions

By Uwe Rudolph¹ and Stephen J. Moss^{2,3,4}

Fast synaptic inhibition in the central nervous system is achieved by the inhibitory neurotransmitter γ -aminobutyric acid (GABA) type A (GABA_A) receptors. These receptors are postsynaptic pentameric complexes that form a central pore that is permeable to chloride ions. GABA typically increases chloride influx, which results in hyperpolarization of the postsynaptic membrane, rendering it less excitable. GABA_A receptors modulate vigilance, emotions, cognition, and muscle tension, and they are the targets of anxiety-reducing and sedative-hypnotic benzodiazepines and some general anesthetics, such as propofol. These drugs increase GABA-induced chloride currents (1). On page 246 of this issue, Han *et al.* (2) describe the identification of an auxiliary protein that interacts with GABA_A receptors and modulates their response to benzodiazepines. These findings have considerable implications for understanding benzodiazepine action because they redefine the components minimally required to support their actions.

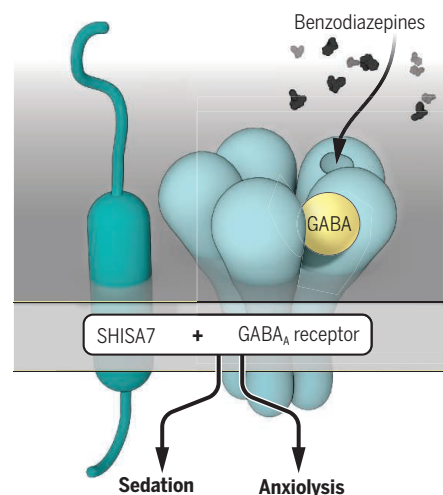
A dynamic balance of excitation and inhibition is required for normal brain function. Neuronal inhibition—through various functionally distinct GABA_A receptor subtypes—sculpts the responses of neurons and neuronal circuits according to environmental influences (1). What are the minimum requirements for drug-induced modulation of synaptic inhibition? Studies in recombinant systems show that expression of GABA_A receptor $\alpha\beta\gamma$ subunit combinations is sufficient for the formation of functional GABA-gated chloride channels that can be modulated by benzodiazepines such as diazepam. Is this also true in vivo? It is apparent that GABA_A receptors perform their physiological functions in concert with other proteins. For example, they

are phosphorylated by tyrosine kinases and by serine-threonine kinases. The lack of phosphorylation-dependent modulation of the $\beta 3$ subunit by mutating two serine residues in the mouse germ line results in deficits in social behavior and increased susceptibility to seizures (3). Moreover, some GABA_A receptor subtypes require interaction with the postsynaptic scaffold protein gephyrin for postsynaptic clustering (4). GABA_A receptor-associated protein (GABARAP) has been shown to link GABA_A receptors to the cytoskeleton, providing a mechanism for cellular targeting and clustering of GABA_A receptors (5, 6). A low micromolar binding of collybistin, a postsynaptic guanine nucleotide exchange factor that regulates the cytoskeleton and connects gephyrin to the GABA_A receptor $\alpha 2$ subunit, has also been identified, and suppression of this binding resulted in loss of a distinct subset of inhibitory synapses, increased anxiety, increased susceptibility to seizures, and early mortality in mice (7).

By contrast, enhancing the interaction between the $\alpha 1$ subunit and collybistin increased the size of inhibitory synapses and

Proteins required for the actions of benzodiazepines

Benzodiazepines exert anxiolytic and sedative actions through γ -aminobutyric acid type A (GABA_A) receptors. Han *et al.* demonstrate that SHISA7 is required for benzodiazepines to exert these actions; the precise mechanisms remain unknown.



¹Department of Comparative Biosciences, College of Veterinary Medicine and Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA. ²Department of Neuroscience, Tufts University School of Medicine, Boston, MA, USA. ³AstraZeneca Tufts Laboratory for Basic and Translational Neuroscience Research, Boston, MA, USA. ⁴Department of Neuroscience, Physiology and Pharmacology, University College London, London, UK. Email: urudolph@illinois.edu; stephen.moss@tufts.edu

synaptic currents, and it reduced seizure sensitivity, demonstrating an important role of collybistin in bidirectionally regulating inhibitory neurotransmission (8). A mutually inhibitory interaction was also suggested between GABA_A receptors and D5 dopamine receptors (9). The GABA_A receptor is also part of a large postsynaptic complex; in one study, 174 GABA_A receptor binding partners, including SHISA7, were identified (10), and it is expected that at least some of these proteins directly interact with GABA_A receptor subunits and modulate GABA_A receptor function.

The SHISA family of transmembrane proteins has been implicated in the modulation of glutamatergic neurotransmission. For example, SHISA6–9 have been shown to regulate α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor function, and SHISA6, SHISA7, and SHISA9 have been identified as AMPA receptor auxiliary proteins that influence trafficking, subcellular localization, and function of AMPA receptors (11). Mice in which the *Shisa7* gene is ablated displayed faster AMPA receptor currents in synapses in the CA1 hippocampal region, as well as deficits in contextual fear memory, whereas auditory fear memory and anxiety-related behavior were normal. Han *et al.* find that SHISA7 is also localized to GABAergic synapses. SHISA7 overexpressed in hippocampal neurons colocalized with GABA_A receptors, vesicular GABA transporter, and gephyrin. An amino-terminal domain within SHISA7, called GABA_A receptor interacting domain (GRID), is required for colocalization with gephyrin and the GABA_A receptor α 2 subunit. In addition, superresolution microscopy analysis suggests that SHISA7 and GABA_A receptors are in close proximity at, or near, inhibitory synapses.

What are the consequences of the GABA_A receptor–SHISA7 interaction? Han *et al.* found that in mice in which *Shisa7* was ablated, GABA_A receptor abundance and channel deactivation kinetics were decreased. The authors evaluated how the lack of SHISA7 expression affects the behavioral response to benzodiazepines. Diazepam reduced locomotor activity, which is indicative of a sedative action, in wild-type mice but not in mice lacking *Shisa7*. Moreover, in an ethological test of anxiety, diazepam displayed an anxiolytic-like action in wild-type mice but not in mice lacking *Shisa7*. Because the reduction of motor activity by diazepam has been shown to be mediated by α 1-containing GABA_A receptors and the reduction of anxiety to be mediated by α 2-containing GABA_A receptors (1)—although not in the CA1 pyramidal neurons that Han

et al. analyzed electrophysiologically (12)—the results indicate that SHISA7 is required for modulation of these receptor subtypes by diazepam. This identifies SHISA7 as a thus far unrecognized essential component required for modulation of these receptor subtypes by benzodiazepines (see the figure).

How SHISA7 exerts its effects on GABA_A receptors remains to be explored, but it could affect the receptors at multiple time points during their life cycle—from their construction in the endoplasmic reticulum to their eventual lysosomal degradation. Given the notable effects of SHISA7 on inhibitory currents, it may regulate the subunit composition of GABA_A receptors, fine-tuning the sensitivity of synaptic currents to benzodiazepine modulation. It remains to be evaluated if SHISA7 interacts with all synaptic GABA_A receptor subtypes and whether it plays a role in regulating extrasynaptic GABA_A receptors, i.e., whether it is a phenomenon specific to selected GABA_A receptor subtypes or universal for all GABA_A receptors.

Han *et al.* use the term “auxiliary subunit,” but this may be confusing because it suggests that SHISA7 is an integral component of GABA_A receptors. However, more than 100 proteins copurify with GABA_A receptors. Disrupting the interactions of two of these proteins, collybistin and/or gephyrin, with GABA_A receptors in mice has more severe effects on inhibitory synapses, even leading to premature mortality, compared with the relatively modest effects of *Shisa7* inactivation. But neither gephyrin nor collybistin are considered to be true auxiliary subunits of GABA_A receptors. Given that SHISA7 is promiscuous, interacting with AMPA receptors as well as GABA_A receptors, it might more accurately be described as an ion channel–preferring auxiliary protein. ■

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MATERIALS SCIENCE

The promise of phase-change materials

Improved nonvolatile memory materials could open up new applications

By Behrad Gholipour

The emergence of artificial intelligence and machine learning techniques and their rapid proliferation across a myriad of industries provide a driving force for improving computing, data storage, and telecommunication hardware architectures. The current generation and use of huge volumes of data will only increase as smart devices, such as autonomous vehicles, utility meters, and medical implants, report out data. To manage this torrent of information, fast data storage and processing devices with low noise and drift that can be manufactured with small physical footprints, in increasingly more intelligent computing architectures,

“Stable phase-change technologies hold the key to major breakthroughs...”

are needed. On page 210 of this issue, Ding *et al.* (1) demonstrate chalcogenide phase-change heterostructures that take advantage of thin phase-change layers interspaced with nanoscale diffusion barriers. The resulting phase-change electronic memories have ultralow noise, drift, and high endurance and may have wide-ranging applications beyond traditional data storage.

Chalcogenide phase-change materials (PCMs) have been a key component in various iterations of optical disk technologies and intensely explored for electronic data storage applications as possible replacements for flash memory (2). Typically, PCMs are made from alloys of sulfur, selenium, and tellurium, the most common being germanium-antimony-tellurium (Ge-Sb-Te, or GST).

Department of Electrical and Computer Engineering, Faculty of Engineering, Donadeo Innovation Centre for Engineering, University of Alberta, Edmonton, Alberta, Canada. Email: bgholipo@ualberta.ca

By applying short optical or electrical pulses with energies as low as femtojoules (3), PCMs can be reversibly switched between a covalently bonded amorphous high-resistance phase and a resonantly bonded low-resistance crystalline phase (see the figure). The crystalline phase also has a higher optical absorption, which, in conjunction with the difference in refractive index between the two phases, is used for switching the intensity of light beams in optical disks (4). These memories are nonvolatile—the states are preserved without the need for external power. Appropriate pulsing protocols for the excitation signal can create intermediate states between the two phases, enabling multilevel operating regimes for both optical and electronic applications (2, 5). The phase transition between the two phases takes place on a femtosecond-to-nanosecond time scale (6, 7), allowing ultrafast switching for potentially more than 10^9 cycles (2).

The reconfigurable functionality of PCMs is now being explored for a variety of computing and telecommunications platforms. Specifically, they can serve as the switchable medium for memristive and neuro-inspired electronic devices (8). In applications such as computing on robotics or sensing platforms, achieving low-power multilevel switching that is fast and reliable, has high endurance, and is comparable in speed to dynamic random-access memory (which has limited data retention and is a volatile memory) is a challenge that is being actively addressed.

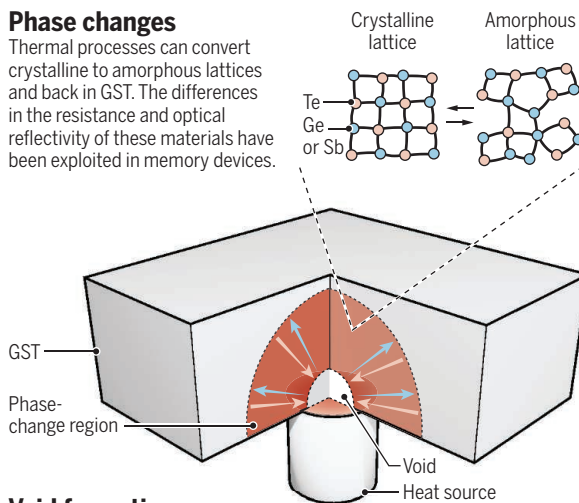
Similarly, PCMs can deliver a variety of reconfigurable optical functionalities such as high-contrast signal-intensity switching, polarization control, and optical beam steering in active plasmonic and photonic metamaterials (9, 10). In such devices, switching over relatively large lateral areas (typically $>20\text{ }\mu\text{m}$) that is reversible and repeatable over many cycles ($>10^{15}$) is desirable for commercial applications. Furthermore, the high optical absorption of PCMs across the visible to near-infrared spectral band can result in reduced switching contrast and increased insertion losses, hindering full-scale commercial uptake of this family of devices for certain applications. This problem is increasingly being addressed through stoichiometric engineering of their optical properties (11, 12). Common to all of these research directions and critical to the continued improvement of existing phase-change random-access mem-

Improving phase-change memories

Ding *et al.* show how interlayer structures can improve the noise and drift performance of germanium-antimony-tellurium (Ge-Sb-Te, or GST) phase-change memories.

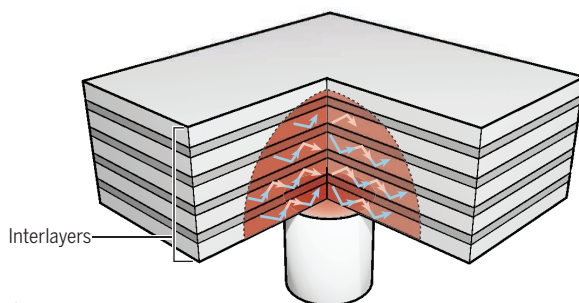
Phase changes

Thermal processes can convert crystalline to amorphous lattices and back in GST. The differences in the resistance and optical reflectivity of these materials have been exploited in memory devices.



Void formation

The local heating of a GST layer can create voids driven by opposing diffusion of Sb and Te (shown by arrows).



Stabilizing layers

Interlayers of TiTe_2 and Sb_2Te_3 hinder diffusion and prevent void formation that degrades performance after repeated phase switching.

ory devices is the requirement for stable (low noise and drift) phase switching in a thermally and chemically stable alloy or device architecture. Such advances would result in reduced switching energies, reliable and repeatable optical and electronic phase contrasts, improved write-erase cycle lifetimes, and faster switching speeds.

To this end, Ding *et al.* report an innovative design alternative for phase-change memory devices consisting of a stacked multilayer structure composed of an antimony-telluride (Sb_2Te_3) phase-change alloy interspaced with titanium-telluride (TiTe_2) confinement layers that act as diffusion barriers (see the figure). Although multilayered superlattices are not a new concept in research on phase-change materials (13), the systematic incorporation of repeated confinement layers based on well-considered design principles presents an important way forward. The proposed structure offers several performance benefits relative to existing designs by reducing

drift in resistance states and noise by suppressing the stoichiometric and structural variability that results from repeated phase-transition cycles in traditional devices without confinement layers.

Many of the challenges addressed by Ding *et al.* will also greatly benefit a number of emerging research directions, including efforts aimed at integrating these materials with various silicon photonics devices for photonic integrated circuits and with optical fibers for spatial-division multiplexing applications (5). The phase contrast in optical properties between amorphous and crystalline phases in PCMs has also recently been explored as switchable color pixels in solid-state displays and as reconfigurable absorptive coatings for adaptive imaging devices (14, 15). They can also be used as switches and routers in emerging quantum computing and telecommunication architectures. Current switching technologies either add too much noise so that single photons are difficult to detect, or they tend to destroy the quantum information. Thus, PCMs with low noise and drift have an as-yet underexplored potential role to play in bringing reconfigurability to this technology platform.

Stable phase-change technologies hold the key to major breakthroughs in a variety of different areas, giving rise to more efficient, smaller, or lighter devices with a myriad of different applications. We cannot foresee which technology platforms will

become the ultimate future of computing and telecommunication. However, high-contrast PCMs that display fast switching with low drift and optical loss will likely be one of the backbones of the adopted platforms. ■

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IMMUNOLOGY

Training T cells for tissue residence

Tissue-specific conditioning of naïve T cells improves targeted immunity

By Donna L. Farber

Immune responses require mobilization of innate cells and lymphocytes in sites of antigen encounter. For T lymphocytes, newly developed naïve T cells populate secondary lymphoid organs such as lymph nodes (LNs), where they become activated by dendritic cells (DCs) presenting antigenic and costimulatory signals. The resultant activated effector cells migrate to tissues to coordinate antigen clearance in situ, after which a subset persists as noncirculating, tissue-resident memory T (T_{RM}) cells. T_{RM} cells are generated in multiple anatomic sites in mice and humans, in response to pathogens, vaccines, allergens, and autoantigens (1). Mouse infection models demonstrate a critical role for T_{RM} cells in protective immunity (2), and T_{RM} cells can promote immunopathology in allergic and inflammatory diseases of the lungs, skin, and gut (3). On page 202 of this issue, Mani *et al.* (4) characterize a pathway of T_{RM} cell development in the skin, which may improve vaccination strategies.

T_{RM} cells express subset-specific molecules that are important for their generation and maintenance. The canonical T_{RM} markers, CD69 and αE -integrin (CD103), both participate in mediating tissue retention of T effector cells; CD69 sequesters sphingosine-1-phosphate receptor-1 (S1PR-1), the surface expression of which is important for lymphocyte egress from the tissue (5), and CD103 binds to E-cadherin expressed by epithelial cells. CD103, in particular, mediates CD8⁺ T_{RM} cell retention in epithelial-rich sites such as skin and intestines (6, 7); this is due to the action of the cytokine, transforming growth factor- β (TGF- β), which can promote CD103 expression on effector T cells that migrate there, and thus facilitate their retention (8). Mani *et al.* reveal a different mechanism by which TGF- β influences CD8⁺ T_{RM} cell establishment in the skin: through direct ef-

fects on naïve T cells that occur in LNs during homeostasis and before exposure to antigen.

Adaptive immune responses originate in LNs, which are adjacent to and drain fluid from various tissue sites through lymphatic vessels. DCs serve as the main cellular conduits by which antigens encountered in tissues, such as skin, intestines, and lungs, are processed, presented, and transferred to tissue-draining LNs for priming of naïve T cells. Migratory DCs can also transmit inflamma-

of heterodimers of the αV subunit and one of five different β chains expressed on the surface of epithelial cells, endothelial cells, fibroblasts, and immune cells. The αV subunit binds LAP and facilitates release of active TGF- β for binding to TGF- β receptors on responding cells (10).

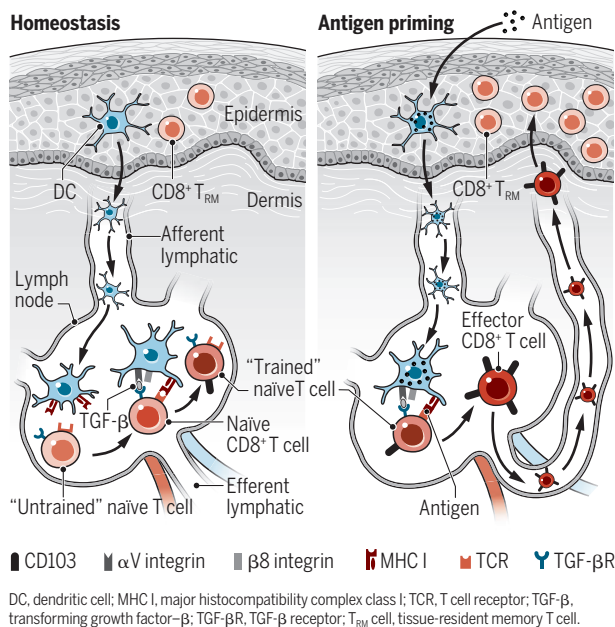
Mani *et al.* investigated the role of TGF- β in the earliest stages of T cell priming by generating mice lacking expression of αV specifically in DCs (αV - Δ DC mice). They found a marked reduction in skin CD8⁺ T_{RM} cells expressing CD69 and CD103 in αV - Δ DC mice compared to wild-type mice in the absence of antigenic challenge. Antigen-specific T cell responses were then investigated by transfer of ovalbumin (OVA)-specific CD8⁺ T cells ("OT-I") into αV - Δ DC or wild-type mice, followed by skin-specific challenge with the antigen OVA. When OVA was introduced 1 day after naïve OT-I transfer, comparable high levels of OT-I T_{RM} cells were generated in the skin of αV - Δ DC and wild-type mice; however, when the OVA challenge occurred several weeks after OT-I transfer, skin OT-I T_{RM} establishment and protective responses were significantly impaired in αV - Δ DC mice. This indicates a requirement for prolonged exposure of naïve T cells to LN DCs (called conditioning), which the authors show involved interactions with major histocompatibility complex class I (MHC I) molecules expressed by DCs that present antigens for T cell recognition by T cell receptors (TCRs). The resultant conditioned naïve T cells acquired CD103 expression and epigenetic modifications that drive gene expression, consistent with a T_{RM} cell-like transcriptional profile.

Therefore, through sustained localization and interaction with DCs, naïve T cells become trained in the LN for future skin residence as T_{RM} cells (see the figure).

Of note, conditioning of naïve T cells occurred over a prolonged period, thus requiring naïve T cells to remain in LNs. Signals for their retention include MHC I, suggesting that naïve T cells perceive cognate TCR signals through weak recognition of self- or cross-reactive epitopes presented by DCs. A previous study demonstrated that naïve CD4⁺

Cellular interactions to condition naïve T cells

During homeostasis, naïve T cells receive training by interactions with DCs. These present active TGF- β and MHC I ligands, upregulate CD103 expression, and undergo epigenetic modifications at T_{RM} cell-associated genes. Upon antigenic priming, trained naïve T cells readily differentiate to skin-homing effectors and CD8⁺ T_{RM} cells in the epidermis.



tory and cytokine-mediated signals important for optimal effector cell differentiation. The essential immunomodulatory cytokine, TGF- β is produced by multiple cell types, particularly in epithelial-rich tissues (e.g., skin, intestines). TGF- β is secreted from cells in a latent complex consisting of homodimers of active TGF- β bound to the latency-associated peptide (LAP) and TGF- β binding proteins associated with the extracellular matrix (9). Activation of latent TGF- β requires binding to certain integrin molecules, consisting

Columbia Center for Translational Immunology, Department of Surgery, Department of Microbiology and Immunology, Columbia University Irving Medical Center, New York, NY, USA. Email: df2396@cumc.columbia.edu

T cell activation can also be influenced by the local LN environment (11). In the study by Mani *et al.*, TGF- β -dependent CD103 expression was induced specifically on naïve CD8⁺ T cells, with no effect on CD4⁺ T cell priming, thereby revealing different requirements for training CD4⁺ and CD8⁺ T cells in LNs. Because CD4⁺ T_{RM} cells are essential for protection from bacterial and viral pathogens, it is important to further dissect pathways that promote their formation.

Studies of T cells in human LNs also suggest long-term retention of naïve T cells and local conditioning. During aging, human LNs are reservoirs for naïve T cells (12). Although a proportion of memory T cells in various human LNs express the T_{RM} cell marker CD69, CD103 is coexpressed specifically in intestinal-draining LNs (13). These LN-specific features are also observed with human DCs, which differentially express tissue-associated markers in LNs draining lungs and intestines (14). Localized adaptations within human LNs can thus influence the maintenance and priming environment for T cells, important for directing T cells to the appropriate tissue site.

Promoting T_{RM} cell formation and tissue immunity at the site of pathogen encounter can have profound benefits in vaccines. Recent preclinical vaccine strategies for site-specific immunity involve two-step “prime and pull” approaches whereby systemic immunization is followed by application of chemokines to a mucosal site (15). The role of chemokines is to attract primed T cells from LNs into the tissue, which may have varying efficacies for recruitment to different sites. The study of Mani *et al.* suggests that introduction of antigen (or DCs preloaded with antigen) to LNs draining a specific tissue site could be a potential one-step strategy for tissue targeting, by engaging local T cells already trained for the task. ■

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NEUROSCIENCE

Linking the need to sleep with synaptic function

Day and night, the amounts of many synaptic proteins vary according to sleep pressure

By Chiara Cirelli and Giulio Tononi

Sleep is essential for the brain: Learning and memory benefit from sleep, whereas sleep loss causes cognitive impairment that can only be reversed by sleep (1, 2). The pressure for sleep increases with time spent awake, and sleep need also depends on the richness of the waking experience and the amount of learning (1, 3). Findings suggest that the restorative effects of sleep are linked to its ability to affect neuronal activity and synaptic plasticity, the capacity to change structure and function based on experience. Synapses are the foundation of neuronal plasticity; in an adult brain, synapses can change their strength and size within minutes or hours in response to new experience and learning (4). On pages 200 and 201 of this issue, Noya *et al.* (5) and Brünig *et al.* (6), respectively, provide evidence that sleep need and synaptic function are tightly linked.

The propensity to fall asleep does not depend only on the duration and intensity of prior waking (homeostatic regulation). Sleep is also under circadian regulation—that is, each of us has a preferred time to go to sleep according to our internal body clock. The two mechanisms are to some extent independent of each other and can be studied separately. Normally, however, they work together to consolidate sleep in one major phase of the 24-hour cycle: the night for humans and the day for nocturnal animals such as mice. The mechanisms responsible for circadian regulation are well characterized at the anatomical and molecular level (7). For example, in both humans and mice there is a master clock located in a single brain area, the suprachiasmatic nucleus of the hypothalamus. This nucleus plays a key role in the daily programming of many organismal functions, from feeding and body temperature to the sleep-wake cycle.

How circadian and homeostatic factors interact to regulate sleep, however, remains unclear. One reason is that a single brain area responsible for the homeostatic regula-

tion of sleep has yet to be discovered. The sleep homeostat may be a distributed system composed of neurons and glial cells such as astrocytes. During waking, such a system may track the duration and intensity of brain activity by sensing cellular signals, including by-products of cellular metabolism such as adenosine. It is unknown how many homeostatic signals exist, how they interact, or how they are transmitted to the specific brain areas that are important to initiate sleep.

The studies of Noya *et al.* and Brünig *et al.* focus on the synapses of the mouse forebrain, the largest, anterior part of the brain that includes the cerebral cortex and the hippocampus. These brain regions are key areas for learning and memory and for many cognitive functions that are affected by sleep deprivation. Together, the two studies offer the first comprehensive overview of how thousands of proteins and their phosphorylation amounts, as well as the messenger RNAs (mRNAs) from which the proteins are transcribed, change across 24 hours in mice. The experiments used biochemical methods to isolate synapses, followed by deep sequencing and advanced mass spectrometry-based proteomics. The results show that synaptic mRNA expression levels undergo daily rhythms, peaking after the mice have been asleep for many hours (around dusk) or at the onset of their waking period (around dawn). A sizable minority of proteins (12%) and more than half of all phosphoproteins also cycle, showing daily rhythms in abundance and phosphorylation status, respectively, and peaking at dusk or dawn (see the figure).

Do these changes in mRNA expression, protein abundance, and protein phosphorylation reflect how long mice were asleep and awake (homeostatic factor), or do they track time across the 24 hours, independent of behavioral state (circadian factor)? Answering this question is not trivial because the propensity to fall asleep depends, at any given moment, on both time of day and duration and intensity of prior waking (8). Noya *et al.* and Brünig *et al.* deprived the mice of sleep for a few hours at different times of day and night to increase sleep pressure to a similar extent across the 24-hour cycle (9).

Department of Psychiatry, University of Wisconsin—Madison, Madison, WI 53719, USA. Email: ccirelli@wisc.edu

The daily oscillations in mRNA expression were either unaffected or reduced (but not abolished) in sleep-deprived mice, indicating that a clock mechanism permits the accumulation of transcripts at specific “rush hours” around dusk and dawn, independent of whether sleep has occurred. Proteins, however, rather than DNA or RNA, carry out most cellular functions. Intriguingly, the daily rhythms of almost all cycling proteins and phosphoproteins were completely abolished when sleep need was kept high, indicating that the cycling of these proteins and phosphoproteins was driven only by sleep and waking, independent of time of day. Of note, the synaptic proteins that show daily

sleep remains incomplete, a clear message from the studies of Noya *et al.* and Brünig *et al.* is that a good place to start looking is the synapse. This conclusion is supported by recent findings of a quantitative phosphoproteomic study performed in whole mouse brain (10). By using two different models of high sleep pressure, the average phosphorylation status of 80 proteins could track changes in sleep need; strikingly, most were synaptic proteins involved in neurotransmitter release and synaptic plasticity. Brünig *et al.* also found that most of these proteins had specific sites with increased phosphorylation at times of high sleep pressure. By looking across the entire 24-hour period, however, they could

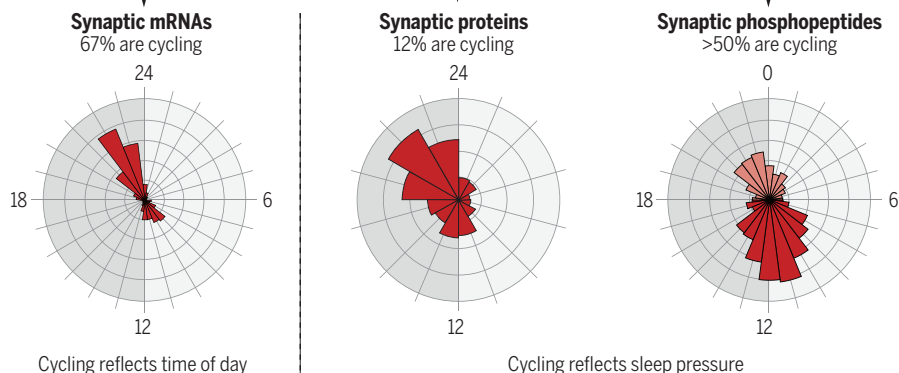
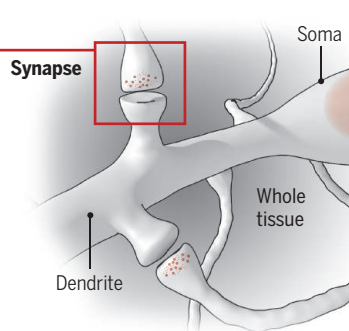
transition to the waking phase (before dusk), whereas several kinases involved in inhibitory activity and synaptic depression become active at the onset of the sleep phase (dawn). This is consistent with overall higher levels of excitatory glutamatergic activity in waking relative to sleeping rodents (11). These data also agree with previous studies that isolated the whole set of synapses from rat cerebral cortex and hippocampus (12) and from mouse forebrain (13) and found a net overall increase in synaptic strength after waking relative to sleep.

The findings of Noya *et al.* and Brünig *et al.* together with previous evidence (10), leave little doubt that synaptic function reflects time spent awake and asleep in mice. One challenge will be to understand how this local signal is translated into sleep, a whole-organism phenomenon. Also, it is unknown which critical aspects of synaptic function require sleep to be restored. Two main hypotheses have been proposed to account for the beneficial effects of sleep on cognition. One idea is that sleep consolidates memories by further strengthening the synaptic connections potentiated by recent learning (1, 14). A competing hypothesis proposes that sleep leads to memory consolidation by promoting broad but selective synaptic weakening (synaptic down-selection) (15). This second mechanism could also explain why sleep can restore the capacity for new learning: by preventing synapses from becoming so strong that they cannot be further potentiated (synaptic saturation). Measuring synaptic function with single-synapse resolution—in many synapses, over many hours of sleep and waking—would be the ideal next step to test these ideas. ■

Synapses keep track of sleep need

In mice, the expression of many synaptic mRNAs and proteins and protein phosphorylation oscillate across the 24-hour period in a distinct manner that is not observed in whole tissue. Dawn and dusk are peak times for both mRNA and protein expression, but their cycling reflects different processes.

- Isolation of synapses
- Purification of synaptic mRNA and proteins
- 24-hour analysis with and without sleep deprivation



changes in abundance differed from those that show changes in phosphorylation levels, implying that both analyses—protein abundance and phosphorylation status—are needed to fully understand how sleep pressure affects synaptic function. Moreover, there was little overlap between the changes in transcriptome, proteome, and phosphoproteome occurring in the synapses and those detected when using whole tissue. This finding reinforces the view that synapses are a special neuronal compartment, affected by circadian and homeostatic factors in distinct ways that differ from those in the cell body of neurons and glial cells.

Although our understanding of the molecular mechanisms that track the need for

also identify many other sites with increased phosphorylation during sleep time.

One of the challenges of omics studies is to provide functional meaning to the long lists of genes and proteins that are identified. As a first step in this direction, Brünig *et al.* focused on kinases (which carry out protein phosphorylation), many of which were found to be enriched in the synapses and modulated by phosphorylation according to sleep pressure. In a few specific cases, they could infer kinase activity from the phosphorylation status of the enzyme. They were thus able to predict whether the enzyme was activated or not. The results suggest that several kinases involved in excitatory activity and synaptic potentiation become active at the

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Young people in Wakiso, Uganda, joined the global climate protests on 20 September 2019.

BOOKS *et al.*

ENVIRONMENTAL POLICY

Confronting climate change

Two authors present the urgent case for a Green New Deal

By **Miriam Aczel**

Proposed by climate activists and supported by U.S. Representative Alexandria Ocasio-Cortez (D-NY) and Senator Ed Markey (D-MA), the “Green New Deal” presents a bold vision for transforming infrastructure at the rate and in the manner that pressing climate change necessitates while restructuring the current economic system. Two new books—Naomi Klein’s *On Fire* and Jeremy Rifkin’s *The Green New Deal*—present arguments for adopting such a plan. Although issuing alarming calls to action, both authors ultimately strike a hopeful note, emphasizing the potential for positive outcomes.

In *On Fire*, writer and activist Naomi Klein distills the development of the climate change mitigation movement over the past decade into a series of thoughtful essays that poignantly highlight the dire situation of the planet. At the same time, she chronicles the unfolding of a “people’s emergency,” characterized by tidal waves of climate-focused civil disobedience.

The largest threats—rising sea levels and increased flooding, droughts and famines, rising global temperatures, and increasing extreme weather events—are also harming livelihoods, deteriorating services and wages,

and disproportionately affecting the poor. We have a “once-in-a-century chance to fix an economic model that is failing the majority of people,” Klein argues. And although the transformation would require ending current consumption patterns, confronting the climate crisis, Klein contends, would lead to creation of millions of good jobs, guaranteed health care, and opportunities for the most “systematically excluded” populations.

A key threat to concerted action is climate skepticism, coupled with a drastic shift in the intensity of emotional responses related to climate issues. When challenging a person’s position on an issue means challenging a central tenet of their identity, facts can be perceived as attacks and are easily deflected. Klein maintains, however, that enhancing communication and tying climate change to other concerns, including the economy and social justice, will help mitigate these threats.

Amid increasing tension between climate advocates and those disavowing climate change, a shift in values is occurring. Today’s activists understand that to change environmental policy requires confronting the values of “rampant greed and individualism” that led to the economic crisis. Social change, Klein contends, begins with radically altering how we relate to each other (and to nature), accepting our collective responsibility to future generations, and respecting the interconnection of all life.

Economic theorist Jeremy Rifkin, whose work has inspired climate legislation in

China and in various countries in the European Union (E.U.), is well positioned to advocate for this new political vision. In *The Green New Deal*, Rifkin confronts skepticism about the feasibility of making a transition of the scale required by countering that we are already making these changes in many global regions. It is time, he argues, for the United States to join the E.U. and China as leaders toward a zero-carbon economy.

Improvising on the title of his influential 2011 climate policy tome (1), Rifkin maintains that we are currently entering the “green digital Third Industrial Revolution,” a paradigm shift in which ownership is replaced with access. The “sharing economy”—made possible by digital infrastructures of energy, communication, and mobility—is, he argues, the first economic system to have emerged since capitalism and socialism. Although the federal government will play a crucial role in framing the transformation, he writes, in today’s increasingly “laterally distributed glocal era,” municipalities and local counties are particularly equipped to play an important role.

Rifkin highlights another potential tipping point, arguing that emerging renewable energies are driving humanity to the “collapse of the fossil fuel civilization.” He cites a 2018 study (2) that concluded that a “carbon bubble”—in which fossil fuel prices will be reduced to compete with renewable prices—would lead to economic and environmental damage if not deflated early. He believes that we can avoid this with rapid decarbonization.

In the second part of the book, Rifkin describes his vision for a Green New Deal in detail, highlighting lessons learned from climate policies in the E.U. and in China. His optimistic call to action is a well-conceived roadmap for how the plan could be deployed, outlining 21 key themes and imperatives, including the need for a carbon tax, the phasing out of fossil fuel subsidies, deployment of 5G broadband and the “internet of things,” tax credits for retrofitting infrastructure with renewable electric heating, the elimination of the sale of internal combustion vehicles, and development of equitable tax laws.

Peer governance will be critical, Rifkin argues, because this next phase will be distributed and will function best if the system remains open and transparent, with operations scaled laterally. If done properly, Rifkin contends that the transition can be achieved in a single generation. ■

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The reviewer is at the Centre for Environmental Policy, Imperial College London, London SW7 2AZ, UK. Email: miriam.aczel14@imperial.ac.uk

AGRICULTURAL POLICY

The precautionary tale of golden rice

A journalist reveals the complicated history of a crop created to help millions

By **Andrew J. Wight**

The term genetically modified organisms (GMOs) inspires images of crazy crops: a single plant that bears tomatoes above ground and potatoes beneath, or a tree that bears a fruit with stripes of yellow sour orange and green stripes from citron. Unlikely as they may sound, the two plants described above are very real, although neither was made in a laboratory. They are products of simple grafting, a technique used by horticulturalists for thousands of years. In *Golden Rice: The Imperiled Birth of a GMO Superfood*, science writer Ed Regis explores why certain food plants are treated by regulatory authorities and the public as “genetically modified,” and therefore worthy of strict cautionary regulation, whereas others are seen as “natural,” despite intensive human intervention in their growth and development.

The book’s title refers to rice whose yellow grains have been genetically altered to express β -carotene to address the widespread problem of vitamin A deficiency, symptoms of which include frequent infections, blindness, and even death. Vitamin A deficiency is estimated to affect one in three children under the age of 5, claiming 670,000 lives every year. The idea behind golden rice was that expressing β -carotene, a vitamin A precursor that occurs in other parts of the rice plant, in the grain, would facilitate the delivery of the vitamin to children in Africa and Southeast Asia, whose main diet is rice.

Golden Rice is a thoughtful and carefully documented tale of how difficult it can be to take something that works in the laboratory and get it to the people who stand to benefit from it. Regis puts his cards on the table from the start, dedicating the book to the scientists who led the development of golden rice. But he also makes clear that these researchers were responsible

for some of the missteps that thwarted the rice’s journey.

In April 1984, at the end of the day at a rice conference, some of the world’s preeminent rice breeders met up for beers and started to discuss new molecular biology technologies, debating which trait they would most like to introduce into rice. The answer, according to veteran rice breeder Peter Jennings, was clear: add a gene for yellow endosperm.

By 2000, Ingo Potrykus and Peter Beyer had identified the right set of genes and inserted them into an existing rice genome (1). Here, Regis gives special mention to Adrian Dubock, an intellectual property expert who negotiated the deals that made



Golden rice (right) is genetically fortified with β -carotene.

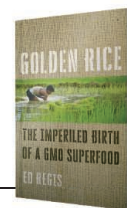
it possible for the researchers to legally use all the patented technologies—from gene constructs to the *Agrobacterium* vector—that had gone into creation of golden rice.

From a scientific standpoint, the technology was a success. So, what prevented golden rice from being quickly disseminated? Although Greenpeace and other anti-GMO activists have been vocal critics of the project, Regis also points to another culprit: the Precautionary Principle.

The Precautionary Principle, cited in the Cartagena Protocol of 2000 (an international agreement on biosafety), allows countries to restrict, postpone, or ban any product or technology without offering any evidence that the item poses a threat or

Golden Rice
The Imperiled Birth
of a GMO Superfood

Ed Regis
Johns Hopkins University
Press, 2019. 256 pp.



danger—“better safe than sorry” writ large. Many years of the Golden Rice Project have thus been spent attempting to comply with stringent regulatory hurdles.

When it came time, in 2012, to transfer the “golden” trait to a strain of rice used in Asia, researchers were forced by the high cost of regulation to select a single cultivar, GRG2. When it produced a lower yield than those of nongolden varieties, the researchers had to make the costly switch to a backup variety in order to ensure that they were bringing to market a grain high in yield and in β -carotene content.

Although nuclear energy has Chernobyl and pharmaceuticals has thalidomide, Regis points out that no such disaster exists for GMOs. (A suspected link between Monarch butterfly decline and Bt corn might have fit the bill, but these claims were later debunked by numerous studies.) While one could argue that it is better to be proactive than to wait for a tragedy to occur before taking precautions, Regis invites

the reader to consider whether it may be worth the unknown risks ostensibly being prevented by regulation to prevent the death and disability that is known to accompany vitamin A deficiency.

After millions of dollars and years of effort, the United States, Canada, Australia, and New Zealand have all recently approved golden rice as safe for consumption. Now, the end goal is in sight: Golden rice is in front of regulators in the Philippines and in Bangladesh, where it is expected to be approved by the end of 2019. ■

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The reviewer is an Australian science journalist based in Medellín, Colombia. Email: at.ligaze@gmail.com



LETTERS

Edited by Jennifer Sills

Grieving environmental scientists need support

Rates of environmental destruction are greater today than at any previous point in human history (1). This loss of valued species, ecosystems, and landscapes triggers strong grief responses in people with an emotional attachment to nature (2). However, environmental scientists are presented with few opportunities to address this grief professionally.

Environmental scientists tend to respond to degradation of the natural world by ignoring, suppressing, or denying the resulting painful emotions while at work (3). The risks that this entails are profound. Emotional trauma can substantially compromise self-awareness, imagination, and the ability to think coherently (4). As Charles Darwin put it, one “who remains passive when overwhelmed with grief loses [the] best chance of recovering elasticity of mind” (5).

Academic institutes must allow environmental scientists to grieve well and thus emerge stronger from traumatic experiences to discover new insights about our rapidly changing world. Much can be learned from other professions in which distressing circumstances are commonplace, such as health care, disaster relief, law enforcement, and the military. In these fields, well-defined organizational structures and active strategies exist for employees to anticipate and manage their emotional distress (6). Effective systems can facilitate healthy grieving

processes, enhance psychological recovery, and reduce the risk of long-term mental health impacts, potentially leading to better practice, decision-making, and resilience in future periods of trauma (7–10). Improved psychosocial working environments for scientists might include systematic training of employees, early-intervention debriefing after disturbing events, social support from colleagues and managers, and therapeutic counseling.

The pervasive illusion that scientists must be dispassionate observers is dangerously misguided. Rather, grief and post-traumatic recovery can strengthen resolve and inspire scientific creativity. To understand and find solutions for our increasingly damaged natural ecosystems, environmental scientists must be allowed to cry and be supported as they move forward.

Timothy A. C. Gordon^{1*}, Andrew N. Radford², Stephen D. Simpson¹

¹Department of Biosciences, Hatherly Laboratories, University of Exeter, Exeter, EX4 4PS, UK. ²School of Biological Sciences, University of Bristol, Bristol, BS8 1TQ, UK.

*Corresponding author. Email: tg333@exeter.ac.uk

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Tibetan hot-spring snakes under threat

Endemic to the Tibetan Plateau, the hot-spring snake (*Thermophis baileyi*) is a relict species that depends on the heat in geothermal zones to survive (1–4). The only snake species found on the Changthang Plateau (5), this sole survivor of the suborder Serpentes (5) has lived for millions of years (2, 3) in natural caves, stone seams, and crannies (6). These shelters, along with wetlands harboring the snake's diet (tadpoles, toads, frogs, and fish) (7), are essential for the survival of the hot-spring snake in this high, cold, and arid habitat.

The local Tibetans believe that hot-spring water can cure many diseases and that the hot-spring snake is the patron saint of these waters [e.g., (8)]. In recent years, most of the hot springs in the areas where the snakes live have been put to use for commercial exploitation (6). Although Tibetans do not directly hurt snakes, their modifications to the natural stone topology and wetland landscape have unwittingly resulted in the loss of the snake's shelters and food (6). In turn, these local snake populations face extinction (6). The construction of roads and thermoelectric plants is even more devastating for the species (6). It is estimated that a total of 15 billion yuan (equivalent to US\$2.1 billion) will eventually be invested in Tibet for the comprehensive exploitation of geothermal resources (9). The hot-spring snake urgently needs help.

It is possible to protect and restore hot-spring-snake populations without compromising the economic development and well-being of Tibetans. To do so, both the government and the public must give more attention and financial support to this species. Scientists must conduct comprehensive and detailed population resurveys, and natural shelters must be protected or artificial snake dens built. Stillwater wetlands should be restored and frogs and toads reintroduced to the habitats. Finally, it will be crucial to monitor the snake population before, during, and after any construction projects involving geothermal resources.

Song Huang^{1,2*} and Lifang Peng¹

¹College of Life Sciences, Anhui Normal University, Wuhu 241000, China. ²College of Life and Environment Sciences, Huangshan University, Huangshan 245041, China.

*Corresponding author. Email: snakeman@sinocephis.com

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10.1126/science.aay9077

Recovered Tibetan antelope at risk again

The Tibetan antelope (*Pantholops hodgsonii*) is an iconic species endemic to the Tibetan Plateau and is the last long-distance migratory ungulate in the region (1). From 1950 to the early 1990s, the species' population declined by 90% due to rampant illegal poaching (2). Thanks to the joint conservation efforts of the Chinese government and international community since the late 1990s, the population has recovered to 200,000 individuals (3). However, the Tibetan plateau is currently experiencing accelerating climate change (4), and the antelope now faces severe climate change-related threats.

Every summer, tens of thousands of antelopes from multiple wintering areas migrate hundreds of kilometers to a shared calving site at Zonag Lake in Hoh Xil Nature Reserve (5). However, in September 2011, a combination of heavy

precipitation, increased glacier melting, and permafrost thawing increased water levels, causing the natural dam containing Zonag Lake to burst (6). The resulting flood formed deep-cutting riverbanks along the traditional antelope migration route, flowing eastward and converging on downstream Yanhu Lake, known for its high salinity (7). A potential spillover of Yanhu Lake is expected in the next 1 to 2 years (6). Meanwhile, the drained Zonag Lake's area is 40% smaller than its original size (8).

These changes to the landscape have serious ramifications for antelope survival. The newly formed riverbanks obstruct the traditional migration route. Antelopes have been observed diverging from the route and are forced to calve along the shore of the downstream Kusai Lake (8). The few individuals that have managed to migrate to the Zonag Lake have had limited access to water surfaces because of its decreased size. Disruptions to migration routes and calving sites are known to cause decreased reproductive success and fitness in ungulate populations (9, 10). If Yanhu Lake were to spill over, the high salinity would lead to catastrophic degradation of the Hoh Xil grassland ecosystem, upon which Tibetan antelopes rely for survival.

Extreme climate-related hazards to wildlife and ecosystems, now occurring worldwide, must be recognized and addressed decisively. To ensure the continued conservation of Tibetan antelopes, the ecological integrity of their current home range must be preserved.

We call on policy-makers to facilitate scientific monitoring and research, explore evidence-based management options, and act swiftly to save one of China's historic conservation success stories.

Jie Pei^{1,2,3}, Li Wang¹, Wenjing Xu³, David J. Kurz³, Jing Geng^{2,3,4}, Huajun Fang^{2,4}, Xinlei Guo^{2*}, Zheng Niu^{1,2*}

¹The State Key Laboratory of Remote Sensing Science, Institute of Remote Sensing and Digital Earth, Chinese Academy of Sciences, Beijing 100101, China. ²University of Chinese Academy of Sciences, Beijing 100049, China. ³Department of Environmental Science, Policy, and Management, University of California, Berkeley, CA 94720–3114, USA. ⁴Key Laboratory of Ecosystem Network Observation and Modeling, Institute of Geographical Sciences and Natural Resources Research, Chinese Academy of Sciences, Beijing 100101, China.

*Corresponding author. E-mail: xinlei.guo@inra.fr (X.G.); niuzheng@radi.ac.cn (Z.N.)

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10.1126/science.aaz2900

TECHNICAL COMMENT ABSTRACTS

Comment on "Cultural flies: Conformist social learning in fruitflies predicts long-lasting mate-choice traditions"

Stephen Thornquist and Michael Crickmore

The claims of Danchin *et al.* (Research Articles, 30 November 2018, p. 1025) regarding long-lasting mate preference based on conformity may result from systematic experimental error. Even if mate copying were a genuine phenomenon, it is unlikely to result in persisting culture in the wild.

Full text: [dx.doi.org/10.1126/science.aaw8012](https://doi.org/10.1126/science.aaw8012)

Response to Comment on "Cultural flies: Conformist social learning in fruitflies predicts long-lasting mate-choice traditions"

Arnaud Pocheville, Sabine Nöbel, Guillaume Isabel, Etienne Danchin

Thornquist and Crickmore claim that systematic experimental error may explain the results of Danchin and colleagues. Their claim rests on mistakes in their analyses, for which we provide corrections. We reassert that conformity in fruitflies predicts long-lasting mate-preference traditions.

Full text: [dx.doi.org/10.1126/science.aaw9549](https://doi.org/10.1126/science.aaw9549)



Tibetan antelopes, a conservation success story, now suffer from climate change–driven habitat disruption.

RESEARCH

IN SCIENCE JOURNALS

Edited by Michael Funk

Artist's rendering
of a neural synapse
with active
protein translation

NEUROSCIENCE

Sleep-wake cycles at mouse synapses

Analysis of the transcriptome, proteome, and phosphoproteome at synapses in the mouse brain during daily sleep-wake cycles reveals large dynamic changes (see the Perspective by Cirelli and Tononi). Noya *et al.* found that almost 70% of transcripts showed changes in abundance during daily circadian cycles. Transcripts and proteins associated with synaptic signaling accumulated before the active phase (dusk for these nocturnal animals), whereas messenger RNAs and protein associated with metabolism and translation accumulated before the resting phase. Brüning *et al.* found that half of the 2000 synaptic phosphoproteins quantified showed changes with daily activity-rest cycles. Sleep deprivation abolished nearly all (98%) of these phosphorylation cycles at synapses. —LBR *Science*, this issue p. 200, p. 201; see also p. 189

STRUCTURAL BIOLOGY

Mastering regulation

The mechanistic target of rapamycin complex 1 (mTORC1) is known as the master kinase, acknowledging its key role in integrating multiple signals to regulate cell growth. When nutrients are abundant,

heterodimers of Rag, a class of small guanosine triphosphatase, bind to mTORC1 and recruit it to the lysosome. Here, other signaling pathways converge on the mTORC1 complex. Anandapadamanaban *et al.* determined cryo-electron microscopy and crystal structures of a RagA/RagC

heterodimer. The structures, together with dynamic studies, explain the nucleotide states required for binding to mTORC1 and support a mechanism for conformational communication between the RagA and RagC subunits in the heterodimer. RagA/RagC binding causes no conformational change in

mTORC1, which is consistent with the idea that mTORC1 must sense additional growth regulators before it is activated. —VV

Science, this issue p. 203

IMMUNOLOGY

Autoimmunity shows its CARDs

Both the adaptor protein CARD9 and loss of the kinase Lyn are associated with autoimmune disease, notably colitis and inflammatory bowel disease. Ma *et al.* found in mice that CARD9 amplified Toll-like receptor signaling and cytokine production in Lyn-deficient dendritic cells but not macrophages. Deleting the *Card9* gene or genes encoding Src-family kinases in dendritic cells prevented the development of Lyn deficiency-associated colitis in mice. Targeting CARD9 or its associated kinases may be a way to relieve inflammation in patients with autoimmune disease. —LKF

Sci. Signal. **12**, eaao3829 (2019).

MATERIALS SCIENCE

Twisting is cool

Rubber bands that are stretched and held in an extended shape for a while will extract heat from their surroundings as they are allowed to relax, owing to a reversal of stress-induced crystallization, which is an exothermic process. Wang *et al.* examine the potential for solid-state cooling of twisted fibers, along with configurations such as supercoiling, for materials including natural rubber, polyethylene, and nickel-titanium fibers. The cooling is related to the change in entropy of the material as it is mechanically deformed. —MSL

Science, this issue p. 216

CANCER

Dishing out treatment recommendations

The number of treatment options for cancer patients keeps expanding, but it remains difficult to predict which tumors will be sensitive to which treatments.

Most patients thus receive treatment according to standardized protocols; some respond to treatment, but others only experience side effects. Ooft *et al.* developed a method of testing drugs in patient-derived organoids, which are biopsy-derived cells from individual patients grown in a dish. In a clinical study, the responses of organoids to the cancer drug irinotecan correlated with patients' responses, suggesting that screening in organoids could help avoid giving irinotecan to patients who would not benefit. —YN

Sci. Transl. Med. **11**, eaay2574 (2019).

ELECTROCHEMISTRY

A direct route to pure peroxide

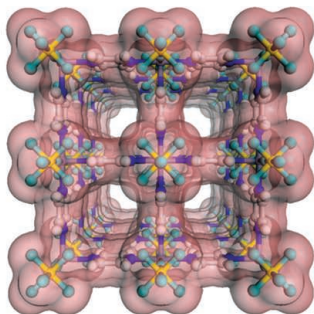
Despite the widespread use of hydrogen peroxide as an oxidant and disinfectant, its commercial synthesis still requires inefficient concentration and purification steps. Xia *et al.* now report an electrochemical approach to synthesizing pure peroxide solutions straight from hydrogen and oxygen. Using a solid-state electrolyte, they avoid contamination of the product solution by extraneous ions. Varying the flow rate of water through the electrochemical cell tunes the final concentration over a range from 0.3% to 20% by weight. —JSY

Science, this issue p. 226

GAS SEPARATIONS

Selecting for ethylene

Purification of ethylene from other gases produced during its synthesis, such as acetylene, ethane, and carbon dioxide, is an energy-intensive process.



Metal-organic framework with molecular selectivity

Chen *et al.* use a mixture of microporous metal-organic framework physisorbents that are selective for one of these four gases. A series of sorbents in a packed-bed geometry produced ethylene pure enough for making polymers. —PDS

Science, this issue p. 241

ECOSYSTEM SERVICES

The future of nature's contributions

A recent Global Assessment by the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services has emphasized the urgent need to determine where and how nature's contribution matters most to people. Chaplin-Kramer *et al.* have developed a global-scale modeling of ecosystem services, focusing on water quality regulation, coastal protection, and crop pollination (see the Perspective by Balvanera). By 2050, up to 5 billion people may be at risk from diminishing ecosystem services, particularly in Africa and South Asia. —AMS

Science, this issue p. 255; see also p. 184

RADIO ASTRONOMY

Probing a galaxy halo with a radio burst

Fast radio bursts (FRBs) are millisecond flashes of radio emission from distant galaxies. It has only recently become possible to locate single bursts precisely enough to determine the host galaxy. Prochaska *et al.* have observed and localized a FRB using a radio interferometer. The line of sight to the host galaxy coincidentally passes through the outskirts of a closer foreground galaxy. By analyzing the propagation of the FRB, the authors put constraints on the density and magnetization of gas in the outskirts of the foreground galaxy. The technique provides complementary information to existing methods using background quasars. —KTS

Science, this issue p. 231

IN OTHER JOURNALS

Edited by **Caroline Ash**
and **Jesse Smith**

PLANT ECOLOGY

Plants, soils, and climate

Environmental change is rarely straightforward in its consequences for natural communities, because of the complexity of spatial and temporal interspecific interactions. Rasmussen *et al.* experimentally studied the effects of temperature and moisture variation on the growth patterns of a perennial herb (*Plantago lanceolata*) and its associated soil microbial community. They used a reciprocal multifactorial design, using plants and soil communities from three different habitats in Sweden. Although warming and increased moisture had a generally positive effect on plant growth, the strength of the response depended on the origin of the plants, as did the responses of root-associated fungi. Thus, climate change may be expected to produce complex patterns of variation in plant-soil interactions, which may be difficult to predict. —AMS

J. Ecol. 10.1111/1365-2745.13292 (2019).



The perennial herb *Plantago lanceolata*

MATERIALS SCIENCE

Glassy carbon for maximum impact

Materials designed for impact absorption need to be able not only to cope with high-stress deformations but also to accommodate high strain, as the energy absorbed is the

integral of the stress-strain response. Although lightweight designed materials, such as those based on trusses, can show high strength or high deformability, they usually cannot do both. Guell Izard *et al.* devised an architected material with smooth interconnected surfaces, similar to a shell, that

CONSERVATION

Species shuffling by humans

Loogeographic regions describe general patterns of faunal organization across the world and our definition of these regions has largely been consistent since the versions put forward by Wallace and Sclater in the mid to late 19th century. As the result of thousands of years of evolution, these regions have largely been thought to be relatively static and resistant to change. Human activities, especially in the 20th and 21st centuries, have caused substantial upheavals in natural systems, leading to the question of whether our activities have also affected these larger faunal associations. Bernardo-Madrid *et al.* surveyed mammals, amphibians, and birds using large datasets and network approaches to determine whether classic associations have been altered. They found that the introduction and movement of species between regions have led to homogenization of many of these regions. Further, predicated extinctions could exacerbate these trends, leading to loss of regional faunal uniqueness. —SNV

Ecol. Lett. **22**, 1297 (2019).



Human activities are resulting in ubiquitous distribution of some species, such as the pigeon, *Columba livia*.

they fabricated out of glassy carbon. Under optimal conditions with a spinodal topology, the propagation of cracks is impeded as they do not align with the stress direction. This enables a slow, progressive failure during impact, and thus higher energy absorption. —MSL

Small **10**, 1002/
smll.201903834 (2019).

BIOTECHNOLOGY

Expanding DNA storage capacity

DNA makes a good archival medium for information storage, thanks to its remarkably high physical density and the relatively low cost of high-throughput sequencing. However, its synthesis presents a challenge both technically and

economically. Two recent studies invented similar methods to augment DNA-based information storage capacity. Instead of using only four standard DNA nucleotides (A, T, G, and C), Choi *et al.* and Anavy *et al.* have developed platforms for reading and writing degenerate nucleotides and mixtures of nucleotides in various predetermined ratios. This expanded alphabet enables higher information content per synthesis cycle. —SYM

Sci. Rep. **9**, 6582 (2019);
Nat. Biotechnol. **37**, 1229 (2019).

NANOMATERIALS

Better contact to WSe₂ nanosheets

The high electron and hole mobility of tungsten selenide (WSe₂) nanosheets should make

them ideal semiconductors for field-effect transistor (FET) devices, but with typical contact materials (silica layers on silicon and gold), mobilities, and on-off ratios fall to impractical levels. Stoeckel *et al.* functionalized one or both sides of WSe₂ single-layer nanosheets with molecules containing silane groups. The tails of these molecules formed monolayers on the nanosheets, whereas the head groups formed monolayers on silica. These molecules also appeared to functionalize defect sites, and their presence changed the work function of the nanosheet and decreased the contact resistance to gold. High mobilities enabled unipolar and, for doubly-functionalized nanosheets, ambipolar FET operation in devices with gold top contacts. —PDS

ACS Nano **10**, 1021/
acs.nano.9b05423 (2019).

MICROBIOME

Antibiotics blunt flu immunity

The microbiome influences our immune system and how we respond to various physiological stresses. Hagan *et al.* report that depleting gut microbiota reduces the ability of influenza vaccine to induce functional immunity in humans. The researchers conducted a small clinical trial of healthy individuals who had low prior exposure either to influenza virus or to the vaccine. One group took a course of broad-spectrum antibiotics before receiving the vaccine, while the other group did not take antibiotics and only received the vaccine. Antibiotic use diminished the gut microbiome composition and impaired the ability of the immune system to generate antibodies. Treatment with antibiotics also disturbed bile acid metabolism and caused inflammatory responses. —PNK

Cell **178**, 1313 (2019).

SCIENTIFIC WORKFORCE

Experience versus salary

Unpaid internships are common in STEM fields, but little is known about the career trajectories of STEM graduates in unpaid versus paid positions. Fournier *et al.* took data from the U.K. Destination of Leavers from Higher Education survey and compared whether science graduates in paid or unpaid positions 6 months after graduating successfully obtained a high salary or were working in a STEM field 3.5 years later. Results show that unpaid work was strongly associated with persistence in STEM but that there is a negative association between unpaid work and future earnings. Personal connections frequently were used by men and high socioeconomic-status graduates to find unpaid work, raising concerns about the diversity of the unpaid workforce. —MMC

PLOS ONE **14**, e0217032 (2019).

ALSO IN SCIENCE JOURNALS

Edited by Michael Funk

CELLULAR NEUROSCIENCE

From trafficking to maintenance

Neurons are remarkably polarized in that proteins made in the cytosol often need to travel many tens or hundreds of cell body lengths along axons to their sites of action in the synapse. Axonal transport of these components is driven by molecular motors along axonal microtubules. Guedes-Dias and Holzbaur review the cell biology of axonal transport and highlight the roles this fundamental process plays in organismal health. —SMH

Science, this issue p. 199

IMMUNOLOGY

Some naïve T cell fates are sealed

Tissue-resident memory T (T_{RM}) cells constitute a subpopulation of memory cells that reside in tissues instead of recirculating. $CD8^+$ epithelial TRM (eT_{RM}) cells, which occupy the epithelium of sites like the skin, require transforming growth factor- β (TGF- β) for their development. Mani *et al.* found that α_v integrin-expressing dendritic cells, which activate and present TGF- β , are key (see the Perspective by Farber). Surprisingly, this interplay did not occur in the skin or draining lymph nodes during T cell priming. Rather, resting naïve $CD8^+$ T cells interacted with α_v integrin-expressing migratory dendritic cells during immune homeostasis, reversibly preconditioning them to become eT_{RM} cells upon activation. A potent cytokine is thus controlled in a context-dependent manner and preimmune T cell repertoires may be less uniform than previously presumed. —STS

Science, this issue p. 202;
see also p. 188

SUPERCONDUCTIVITY

Modulating superconductivity

Strain can have considerable effects on the electronic properties of materials. For instance, the temperature at which a material becomes superconducting—the critical temperature—can be tuned by varying strain. Bachmann *et al.* used focused ion beam milling to fabricate microstructures of the superconductor $CeIrIn_5$ on sapphire substrate. A difference in the thermal contraction coefficients of the two layers induced nonuniform strain upon cooling of the sample, leading to large gradients of the critical temperature. This approach can be used in other materials and may enable fabrication of superconducting circuitry without physical junctions. —JS

Science, this issue p. 221

SUPERCONDUCTIVITY

Unconventional oscillations

At sufficiently low temperatures, superconductors expel an applied magnetic field. However, if the topology of the superconductor is nontrivial—for example, if there is a hole in the sample—there can be a nonzero magnetic flux inside the hole. This flux can only take certain discrete values, and the superconducting critical temperature has maxima at the corresponding values of the magnetic field. Li *et al.* studied these so-called Little-Parks oscillations in superconducting rings made out of polycrystalline thin films of β - Bi_2Pd . They found that the phase of the oscillations was shifted by π compared with oscillations observed in most superconductors, as predicted for certain unconventional pairing symmetries. —JS

Science, this issue p. 238

SURFACE CHEMISTRY

Imaging a chemisorption process

At low temperatures, a molecule may adsorb to a surface only through weak forces (physisorption), and only upon heating and overcoming an energetic barrier does it form a strong covalent bond (chemisorption). Huber *et al.* imaged this transition for an atomic force microscopy tip terminating in a carbon monoxide molecule. Although the oxygen atom of the tip is normally considered to act like a rare gas atom, interacting only through van der Waals interactions, at short distances directly above a transition metal atom, it transitions to a strongly interacting chemisorption state. —PDS

Science, this issue p. 235

NEUROSCIENCE

Fine control of brain GABA_A receptors

GABA_A (γ -aminobutyric acid type A) receptors are ligand-gated anion channels that mediate fast inhibitory transmission in the mammalian brain. Han *et al.* investigated if a protein called SHISA7 was involved in regulating GABA_A receptor expression and function (see the Perspective by Rudolph and Moss). SHISA7 was found at GABA-releasing synapses, where it interacted with GABA_A receptors, controlled receptor concentration at the synapse, sped up receptor deactivation kinetics, and modulated some behavioral properties of benzodiazepines. SHISA7 thus affects surface expression, gating kinetics, and pharmacology of GABA_A receptors in the brain. —PRS

Science, this issue p. 246;
see also p. 185

NEUROSCIENCE

An inhibitor causes neuronal excitation

Glycine is thought to be primarily an inhibitory neurotransmitter. However, it also acts as a coagonist on excitatory *N*-methyl-D-aspartate (NMDA) receptors. Otsu *et al.* examined the function of the NMDA receptor subunit combination GluN1/GluN3A in the medial habenula (MHb) of adult mice. This NMDA receptor subunit combination in MHb neurons is activated by glycine released from astrocytes. Activation of GluN1/GluN3A NMDA receptors causes depolarization and increased spiking of MHb neurons. Reducing GluN3A receptor subunit levels in the MHb blocks conditioned place aversion. —PRS

Science, this issue p. 250

INNATE LYMPHOID CELLS

Keeping track of time

A number of bodily functions, from feeding to sleeping, are regulated by circadian clocks. Teng *et al.* and Wang *et al.* report that expression of several genes in intestinal group 3 innate lymphoid cells (ILC3s) in mice is synchronized to the time of day. To probe the role of clock proteins in regulating ILC3 functions, the two groups studied mice lacking distinct clock proteins, BMAL1 and REV-ERB α . The loss of these clock proteins resulted in decrease in the number of intestinal ILC3s, but these cells produced higher levels of certain cytokines. How clock proteins fit within the larger network of transcription factors regulating development and gene expression in ILC3s remains to be seen. —AB

Sci. Immunol. **4**, eaax1215,
eaay7501 (2019).

PHASE-CHANGE MEMORY**Getting more bits
out of PCRAM**

Phase-change random access memory (PCRAM) has the ability to both store and process information. It also suffers from noise and electrical drift due to damage that accumulates during the cycling process. Ding *et al.* developed a phase-change heterostructure where a phase-change material is separated by a confinement material, creating an alternating stack (see the Perspective by Gholipour). This architecture results in ultralow noise, lower drift, and stable multilevel storage capacity, which are potentially useful for new forms of computing. —BG

Science, this issue p. 210;
see also p. 186

REVIEW SUMMARY

CELLULAR NEUROSCIENCE

Axonal transport: Driving synaptic function

Pedro Guedes-Dias and Erika L. F. Holzbaur*

BACKGROUND: Neurons are polarized cells with extreme geometries. Multiple dendrites and one axon generally emerge from a single cell body and establish synaptic contacts with their partners. Synaptic maintenance and plasticity rely on the active delivery of newly synthesized components to these sites, which may be localized up to a meter from the cell body. Cargos that are actively trafficked along the axon include synaptic vesicle precursors, mitochondria, signaling endosomes, autophagosomes, lysosomes, and mRNA granules. In axons, these cargos use cytoplasmic dynein and kinesin motors to navigate a uniformly polarized microtubule network to reach their destinations. In dendrites, microtubules are organized in a more complex, bipolar pattern that is effectively

navigated by a distinct subset of neuronal motor proteins and is less understood overall.

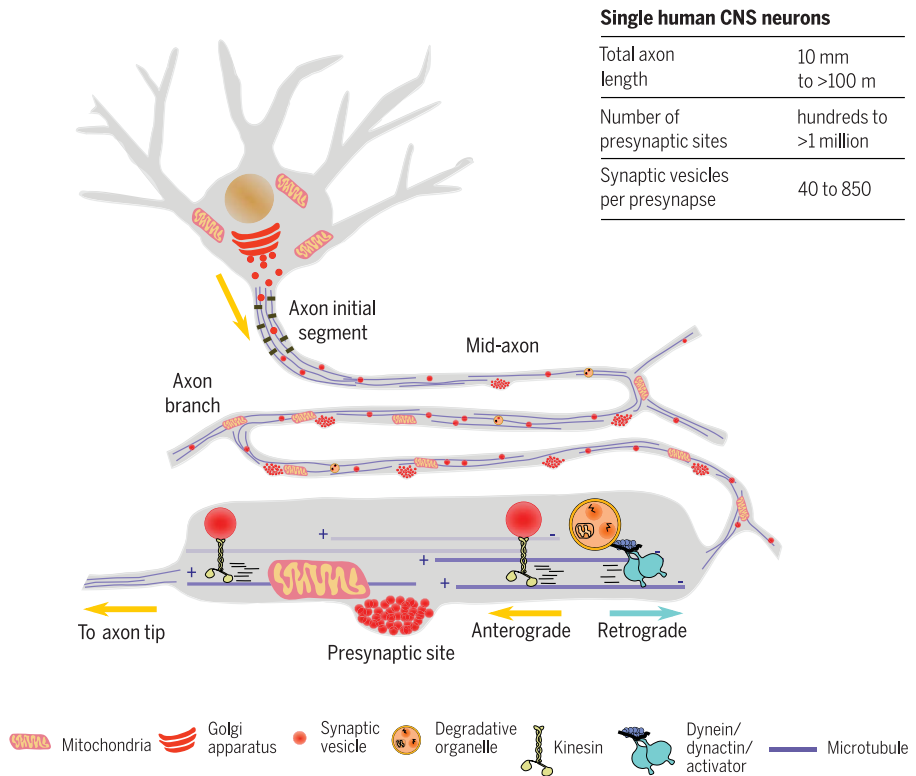
ADVANCES: Traditionally, the meter-long axon of human lower motor neurons has been considered as an example of the long distances cargo must be conveyed from soma to synaptic terminal. Advances in connectomics and axonal tracing techniques are providing us with an increasingly accurate depiction of the morphology and size of axonal arbors in the central nervous system (CNS) and the many synaptic connections that mediate neuronal function. In humans, it is estimated that the axonal arbor of some neuronal populations in the CNS can range up to hundreds of meters in total length and may contain thousands of “en passant” synapses along its extent. Im-

proved and complementary in vitro and in vivo imaging approaches are now allowing the elucidation of increasingly intricate mechanisms by which the activities of dynein and kinesin motors regulate organelle transport along axons. Recently, efforts have focused on identifying the adaptor proteins that specify motor-cargo selectivity and the regulatory mechanisms that govern the directed transport of cargo-carrying opposing motor proteins. In parallel, important advances are being made in our understanding of how the axonal microtubule network is organized and how changes at the microtubule level can affect motor activity to finely regulate axonal transport. A picture is now emerging whereby the exquisite interplay of several regulatory mechanisms functioning at multiple levels directs how and when motors initiate and terminate transport, and how molecular motors respond to local cues to deliver cargo with high precision along the axon. This type of targeted delivery is required to maintain essential neuronal functions such as synaptic activity.

OUTLOOK: The intracellular transport system in neurons is specialized to an extraordinary degree, enabling the delivery of critical cargo to sites in axons or dendrites that are far removed from the cell center. Distance is not the only challenge. Localized delivery of presynaptic components provides another layer of complexity that must be successfully navigated to maintain synaptic transmission. Innovative approaches to determine the mechanisms regulating axonal transport and cargo delivery, the number and lifetime of presynaptic components, and the metabolic requirements to maintain synaptic activity are required. These advances will be key to assembling a more comprehensive and quantitative framework of axonal transport and its central role in presynaptic operation.

A growing number of mutations across the molecular machinery involved in axonal transport are being identified that cause a range of neurodevelopmental and neurodegenerative diseases. Both nerve injury and chemotherapy can also disrupt trafficking pathways in neurons. Hopefully, the mechanistic insights being developed now will provide a framework for the design of successful therapeutic interventions for both genetic and trauma-induced disruptions in axonal transport and synaptic function. ■

The list of author affiliations is available in the full article online.
*Corresponding author. Email: holzbaur@penmedicine.upenn.edu
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Axonal transport drives cargo through extreme geometries. Neurons in the human central nervous system display highly complex axonal arbors that can branch thousands of times, reach hundreds of meters in total length, and contain hundreds of thousands of presynaptic sites distributed “en passant.” The axonal transport machinery supports synaptic function by delivering new synaptic vesicles to and removing aged organelles from presynaptic sites.

REVIEW

CELLULAR NEUROSCIENCE

Axonal transport: Driving synaptic function

Pedro Guedes-Dias^{1,2} and Erika L. F. Holzbaur^{1*}

The intracellular transport system in neurons is specialized to an extraordinary degree, enabling the delivery of critical cargo to sites in axons or dendrites that are far removed from the cell center. Vesicles formed in the cell body are actively transported by kinesin motors along axonal microtubules to presynaptic sites that can be located more than a meter away. Both growth factors and degradative vesicles carrying aged organelles or aggregated proteins take the opposite route, driven by dynein motors. Distance is not the only challenge; precise delivery of cargos to sites of need must also be accomplished. For example, localized delivery of presynaptic components to hundreds of thousands of “en passant” synapses distributed along the length of a single axon in some neuronal subtypes provides a layer of complexity that must be successfully navigated to maintain synaptic transmission. We review recent advances in the field of axonal transport, with a focus on conceptual developments, and highlight our growing quantitative understanding of neuronal trafficking and its role in maintaining the synaptic function that underlies higher cognitive processes such as learning and memory.

Presynaptic maintenance and plasticity require the delivery of newly synthesized cargo originating from the neuronal cell body, as well as the clearance of aged synaptic components. Both supply and clearance require that organelles effectively navigate long distances and the extreme geometries that axons can display in vivo. With our improved understanding of the dimension, complexity, and organization of the axonal arbor of some neuronal populations in the central nervous system (CNS), there is a concurrent evolution of the concept of axonal transport as a central mechanism for axonal function and presynaptic operation. Although experimental progress has begun to disentangle the “distance to travel” problem, new questions have emerged regarding how the transport of multiple cargos is specifically regulated to maintain the numerous presynaptic sites along the axon, each properly supplied with all the necessary components critical to ensure reliable neurotransmission. Thus, the effective trafficking of cargos within the neuron is the baseline requirement for the function of neuronal circuits, such as the ability to learn and create memories.

Microtubules and motors

Microtubules are the tracks used by cytoplasmic dynein and kinesin, the molecular motors that drive long-distance transport in neurons. Microtubules are formed from the head-to-tail polymerization of α - and β -tubulin heterodimers

into protofilaments that assemble laterally to form a hollow tube. Neuronal microtubules are generally composed of 13 protofilaments, although differences exist among species and neuron types (1). Because α -tubulin heterodimers are assembled with the α -tubulin facing one end (minus-end) and β -tubulin the other (plus-end) of the microtubule, the overall polymer structure is polar. The microtubule minus-end region is relatively stable, whereas the plus-end can be more dynamic, actively growing and shrinking by addition and loss of tubulin heterodimers (2).

Microtubule-based motors are essential for the major long-range transport of cargos along the axon, moving over distances of up to a meter and speeds of 1 to 3 $\mu\text{m/s}$. A superfamily of more than 45 kinesins is expressed in mammals, grouped into distinct families according to sequence homology and structural similarities. Motors from four of these families—kinesin-1, kinesin-2, kinesin-3, and kinesin-4 family motors—traffic cargos along the axon in the anterograde direction, moving outward from the soma. Other kinesins have been implicated in microtubule organization and remodeling (3). In contrast to this diversity, the retrograde transport of cargos toward the soma is driven by a single motor, cytoplasmic dynein. Dynein's specificity for cargo is regulated by a broad group of activating adaptors (4). Myosin motors moving along actin filaments contribute to short-range cargo movement in neurons, particularly at synapses (3); it is becoming clear that microtubule- and actin-based dynamics tightly interact and that this interplay has important roles, especially in neural development and regeneration.

Axonal transport is typically divided into “fast” and “slow” components. The fast component includes the transport of vesicular

cargo and organelles and generally occurs at relatively high velocity ($>0.5 \mu\text{m/s}$), whereas the slow component includes the transport of soluble cargo such as synapsin or filamentous cargo such as neurofilaments and is much slower ($<0.1 \mu\text{m/s}$) (5). Reliable transport and accurate delivery of fast-moving cargo to specialized regions in the axon relies on the integration of multiple internal and external signals that ultimately affect the motor-microtubule interaction. The multiple mechanisms mediating the axonal transport of cargo functionally converge to ensure the maintenance of presynaptic homeostasis by replenishing presynapses with fresh or additional proteins or organelles (such as synaptic vesicles and mitochondria) and removing old and defective synaptic components (6).

Axonal microtubule polarity

A characteristic feature of the axonal microtubule network is its “plus-end out” arrangement (7–9). It is not yet fully clear how axonal microtubule polarity is specified and maintained, but the formation and integrity of the axon initial segment (AIS) is emerging as an important determinant of this process. The AIS acts as a barrier separating the somatodendritic and axonal compartments (10). Dynein activity in the AIS is critical for maintaining axonal identity and microtubule polarity (11–13). The critical AIS component ankyrin G interacts indirectly with peripheral AIS microtubules through end-binding (EB) proteins (14), which in the AIS are bound all along the microtubule lattice (14) rather than binding specifically to dynamic microtubule plus-ends, as is more commonly observed throughout the neuron. The microtubule cross-linkers MTCL1 (15) and TRIM46 (16, 17) also localize to the AIS. MTCL1 maintains ankyrin G localization (15) and TRIM46 is a microtubule cross-linker that bundles parallel microtubules (16, 17). TRIM46 knockdown induces a loss of microtubule polarity within the axon (16), which suggests that the integrity of the AIS is critical to maintaining the uniform microtubule polarity found throughout the axon.

The selective sorting of parallel microtubule cross-linkers to the axon can potentially facilitate the maintenance of the uniformly polarized microtubule network in this compartment. Early work has shown that before axon specification, the microtubule network is uniformly plus-end out in all minor neuritic processes (18). Selective microtubule stabilization in one minor process determines axon specification (19), and only after this event does the dendritic microtubule network polarity become progressively mixed (18). CAMSAP2, a microtubule minus-end-binding protein that stabilizes microtubules (20, 21), plays an important role in specifying neuronal polarity (22). Interestingly, CAMSAP2 accumulates proximally

¹Department of Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.

²Institute of Neuronal Cell Biology, Technische Universität München, 80802 Munich, Germany.

*Corresponding author. Email: holzbaur@pennmedicine.upenn.edu

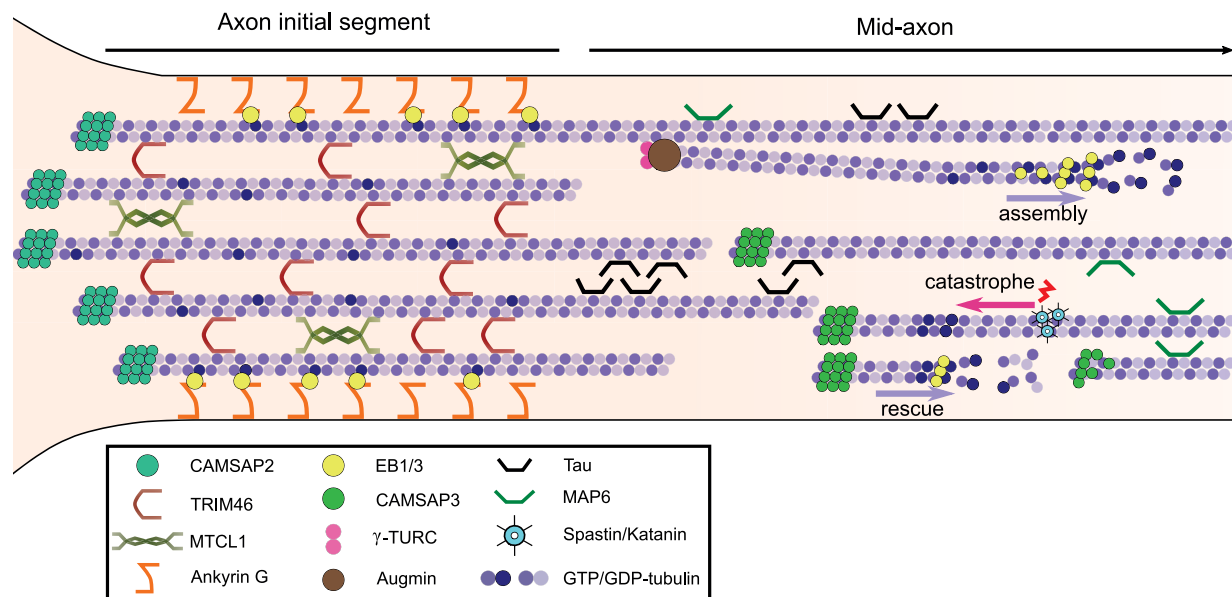


Fig. 1. Axonal microtubule organization and polarity. Stabilization of the microtubule “plus-end out” arrangement in the axon initial segment (AIS) is critical for axonal identity and likely acts as a template to establish the uniform polarity of the axonal microtubule network along the mid-axon.

to the AIS in developing neurons (22). It is conceivable that in the early stages of axonal differentiation, axonal microtubule polarity is specified by the selective bundling of plus-end-out microtubules in the AIS by TRIM46 and the minus-end stabilization of the bundled microtubules by CAMSAP2. This initial microtubule array could thus provide the template from which a uniformly polarized microtubule network can extend along the axon (Fig. 1).

Extension of the axonal microtubule network

Axonal elongation requires the persistent generation of properly oriented microtubules. In neurons, the centrosome gradually shuts down during development and neurite extension is sustained through acentrosomal microtubule nucleation (23) via the γ -tubulin ring complex (γ TuRC) (22, 24). Augmin has recently been identified as an important factor for steering the polarity of axonal microtubule nucleation (24). Augmin recruits γ TuRC to microtubule lattices to nucleate microtubule branches and, together with NEDD1, likely controls the geometry of microtubule network assembly (24). Recently, SSNA1 was reported to induce branched microtubule polymerization (25). SSNA1 localization at axonal branch points (25) suggests that it may play a role in specifying microtubule plus-end-out polarity to newly formed axonal branches. Microtubule plus-end guidance into axonal branches is assisted by the actin cytoskeleton via interactions with septin 7, which is located at the base of filopodia (26), and drebrin, which is located along the proximal region of filopodia (27).

Stabilization of newly formed microtubules is required to maintain axonal polarity. Mid- and distal axonal microtubules can be stabilized

by the minus-end-binding protein CAMSAP3 (28). The microtubule network along the axon can be further stabilized by interacting with endoplasmic reticulum (ER) tubules, likely through direct binding of the ER-shaping protein P180 to the microtubule lattice (29). Microtubule-associated proteins (MAPs), including MAP6 and Tau, also stabilize axonal microtubules and assist the formation of axonal microtubule bundles (30, 31). In contrast to TRIM46 in the AIS (16), Tau does not have intrinsic parallel microtubule cross-linking properties (32). Nonetheless, evidence suggests that Tau-mediated bundling of distal microtubules is important to steer microtubule polarity and growth during axonal development (33).

The microtubule-severing activity of katanin and spastin has been shown to amplify microtubule mass in vitro (34, 35). Microtubule severing followed by growth of the two resulting polymers can potentially assist in maintaining axonal microtubule polarity (36, 37). However, this mechanism has not yet been directly demonstrated in neurons. In fact, microtubule-severing function in neurons in vivo remains ambiguous. Spastin knockout in *Drosophila* led to a decreased microtubule density at terminal neuromuscular junction (NMJ) synapses (38), whereas spastin knockout mice showed the opposite phenotype with increased axonal microtubule mass in NMJs (39). Future studies will be required to elucidate the roles of microtubule-severing enzymes in axonal and presynaptic microtubule organization.

Axonal microtubule organization

The results of multiple electron microscopy (EM) studies agree that axonal microtubules

are discontinuous structures. Estimates of microtubule length range from 0.05 to 40 μ m in developing axons of cultured hippocampal neurons (40), and up to several hundred micrometers in some neuronal subtypes (41–43). These individual microtubules are organized to form a tiled array along the axon, allowing for continuous transport of cargos from soma to axon tip (or from tip to soma) by kinesin and dynein motors. In myelinated axons, EM work has shown that microtubules are mostly continuous through the nodes of Ranvier (42). In these specialized regions of shortened axonal diameter, the microtubule number remains virtually unchanged but the microtubule bundle is more compacted (42) and contains fasciculated microtubules, a feature that was previously thought to be unique to the AIS (44).

Technical challenges have thus far curtailed a thorough characterization of the axonal microtubule network, particularly at presynaptic sites. However, live fluorescence imaging approaches have been applied recently to investigate axonal microtubule organization. A novel approach based on the quantitative analysis of fluorescently tagged patronin/CAMSAP and tubulin was successfully applied to quantify microtubule number, length, and spacing along the axon in *Caenorhabditis elegans* neurons (45). This strategy confirmed the seminal findings of Chalfie and Thomson, who investigated microtubule organization using EM (46), with the additional advantage that Yogeve *et al.* (45) were able to spatially localize microtubule plus- and minus-ends within the tiled microtubule array of the axon while simultaneously monitoring the dynamicity of individual microtubules. This approach, together with other studies using fluorescence-based imaging

techniques, including spinning-disk confocal microscopy (47) and super-resolution microscopy (48), also confirmed that microtubules are more densely packed in the axon shaft and become sparser and more played at growth cones and axonal termini. Distal microtubules are more dynamic and enriched in tyrosinated α -tubulin (48), a marker of newly polymerized microtubules (see below and Box 1). Both the lower microtubule density and more dynamic nature of these microtubules have important implications for the initiation of retrograde transport, as detailed below.

Analysis of microtubule dynamics in primary hippocampal neurons revealed that end-binding protein 3 (EB3) puncta initiate and terminate preferentially near or at presynaptic sites, indicating that synaptic regions show higher microtubule dynamicity than nonsynaptic regions

along the axon (49). These findings indicate that en passant synapses along the axon are enriched in highly dynamic microtubule plus-ends. An analogous microtubule organization was described in peripheral motor neuron axons of adult mice, where EB3 dynamics were found to be higher in the presynaptic areas of the NMJ than in the nonsynaptic intercostal sections of the axon (50). Together with previous EM work showing that microtubules in synaptic areas of olfactory axons are shorter than microtubules in the nerve proper (43), a picture of how the axonal microtubule network is organized is emerging. These studies suggest that long microtubules support efficient fast transport of vesicles along the axon, whereas in synaptic areas, shorter, more dynamic microtubules promote cargo pausing and local delivery to presynaptic sites.

Axonal microtubule diversity

Microtubules provide the guiding tracks for motor movement along the axon, influencing motor activity through multiple levels of regulation. The tubulin nucleotide state, microtubule posttranslational modifications (PTMs), and tubulin isoform composition act in a concerted way to create cytoskeleton specializations and locally regulate transport (Fig. 2).

Nucleotide state of tubulin

Guanosine triphosphate (GTP)-bound tubulin heterodimers incorporate into the growing tip of a microtubule. Tubulin polymerization and incorporation into the microtubule lattice catalyzes hydrolysis of the GTP bound to the β subunit (51). This hydrolysis changes the conformation of the mammalian microtubule lattice from an expanded to a compacted state (52), influencing the affinity of several microtubule-binding proteins. For example, EB proteins have a higher affinity for tubulin in a GTP-bound state, and this underpins the dynamic recruitment of EBs to the growing microtubule tip where they regulate microtubule dynamics (53). In contrast, the lower affinity of the kinesin-3 motor KIF1A for GTP-tubulin facilitates release of this motor and its cargo from the microtubule upon encountering a dynamic microtubule plus-end (49).

For decades, the prevalent view was that GTP-tubulin incorporation was confined to the microtubule tip. However, this idea has recently been challenged by a series of elegant *in vitro* studies (34, 54, 55). This work shows that lattice defects originating from either mechanical stress or enzymatic activity can be rescued by the local incorporation of free

Box 1. Challenges in tackling the nanoscale distribution of tubulin modifications and isoforms.

Neuronal microtubules are highly modified and are likely an ensemble of multiple tubulin isoforms; however, the spatial distribution of tubulin modifications and isoforms along the neuronal cytoskeleton at the nanoscale level is still unclear. Most of what is known about microtubule modifications in neurons stems from the use of a handful of antibodies that target acetylated, tyrosinated/detyrosinated, and glutamylated (≥ 1 glutamate-long side chain) and polyglutamylated tubulin (≥ 3 glutamate-long side chain). Generally, these antibodies have a size of 15 nm, and their optical detection requires binding with a secondary antibody of a similar size, resulting in a ~ 30 -nm complex. Given that tubulin subunits are 4 nm apart, this approach is not suitable to resolve small-scale modifications. The problem is further compounded by the packed arrangement of the axonal microtubule array. Nano-antibodies to tubulin have been developed (194). Further increasing the repertoire to target different tubulin modifications and isoforms, and combining it with improved super-resolution microscopy techniques such as expansion microscopy or the recently developed motor-PAINT (195), will reveal a more detailed picture of tubulin diversity along the axon and help to clarify how it affects the activity of motor proteins.

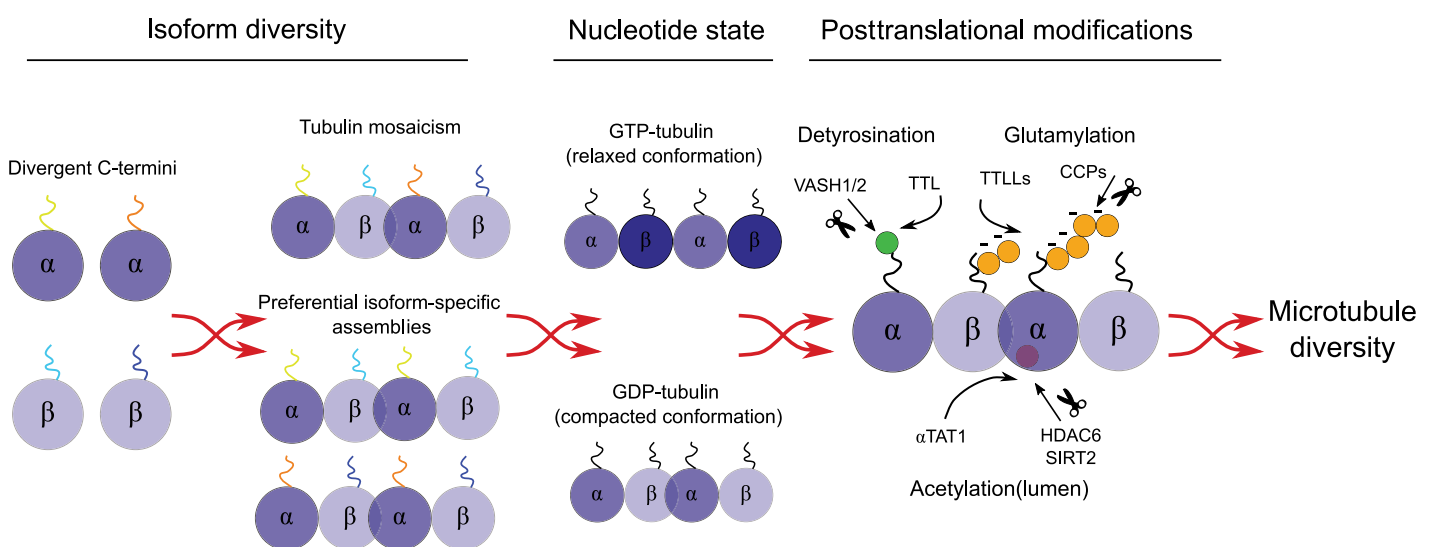


Fig. 2. Axonal microtubule diversity. From numerous tubulin isoforms to distinct nucleotide states and several PTMs, microtubule lattices can in theory display a large number of combinatorial arrangements. This diversity can presumably be shown either at the single-microtubule level (multitude of tubulin isoforms, nucleotide states, and PTMs distributed across the same microtubule lattice) or at the microtubule population level (uniformity of tubulin isoforms, nucleotide state, and PTM distribution across microtubule populations, possibly conferring these a specific function).

tubulin, presumably in the GTP state. The controlled generation of GTP-tubulin islands along the microtubule lattice may locally regulate the function of specific microtubule effectors. Indeed, GTP-rich microtubules were detected in the AIS (56), where EB proteins are known to bind the length of microtubules (14), and at en passant synapses, where GTP-rich microtubules specify synaptic cargo delivery (49). It is unclear from these in vitro studies (34, 54, 55) whether and at what point the incorporation of GTP-tubulin into the microtubule lattice is followed by GTP hydrolysis. Nonetheless, a system whereby microtubules undergo self-renewal through the incorporation of free tubulin heterodimers into a pre-existing polymer could potentially extend the lifetime of a single microtubule indefinitely, akin to a “ship of Theseus” paradox. Notably, by bypassing the need to depolymerize the long axonal microtubules, this model would allow the continuous renewal of the microtubule network without interrupting active transport. Moreover, GTP-tubulin islands can create points of microtubule rescue and regrowth (54, 57, 58) and may be the mechanism underlying the high local microtubule dynamicity at presynaptic sites. Although incorporation of photoconverted tubulin into a pre-existing microtubule lattice has been reported in cells (54), the extent to which cells (and, in particular, neurons) rely on this mechanism to maintain their microtubule network remains unclear and needs to be further investigated.

Additional mechanisms may also affect the lattice structure of the microtubule, and thus the relative binding affinity of molecular motors and other microtubule-associated proteins. For example, recent work indicates that the binding of the motor protein kinesin-1 to guanosine diphosphate (GDP) microtubules is sufficient to induce the expansion of the microtubule lattice to a pseudo-GTP state (59, 60). This change in conformation is cooperative, with a ~10% binding saturation of a kinesin-1 motor domain predicted to be sufficient to expand the lattice across the whole microtubule (60). However, in vivo, the binding of kinesin-1 to microtubules is tightly regulated by auto-inhibition, so it will be important to assess the possible impact of this mechanism on the binding of other motors or effectors that are sensitive to the nucleotide and conformation states of the microtubule lattice.

Posttranslational modifications of tubulin

Tubulin can be subjected to a wide range of PTMs. Acetylation, detyrosination, and polyglutamylation are particularly prevalent in axonal microtubules (61).

α -Tubulin is acetylated in K40 by α -tubulin acetyltransferase (α TAT1) and deacetylated by histone deacetylase 6 (HDAC6) and sirtuin 2 (SIRT2) (62). It has long been proposed that

α -tubulin acetylation regulates axonal transport, although the exact mechanism whereby the acetylation of a luminal tubulin residue can modify motor activity remains elusive (62). Recently, it was shown that acetylation increases microtubule resistance to mechanical breakage (63). Tubulin acetylation might be especially important for increasing the longevity of axonal microtubules given their length and shape, which may be bent at axonal turns or by the sudden migration of microglia through the neuropil (64). Studies suggest that tubulin acetylation may also play a role in specifying proper axonal pathfinding. Loss of α -tubulin acetylation increased axonal microtubule dynamics (65), leading to excessive axonal branching in the cortex (65, 66) and distortion of the dentate gyrus in mice (67). Although spatial learning and memory were not impaired, mice in which α -tubulin acetylation was disrupted displayed increased anxiety-like behavior (65).

Detyrosinated microtubules are thought to be longer-lived than microtubules composed of tyrosinated α -tubulin, as the removal of the terminal tyrosine residue preferentially occurs once α -tubulin subunits are incorporated into polymer (68, 69), while retyrosination occurs on free tubulin subunits (70). There is a microtubule detyrosination gradient running down the axon of developing sensory neurons, with newer tyrosinated microtubules enriched at the distal axon (48). Tyrosination is enriched at the tips of growing microtubules (70) and in regions with high microtubule dynamicity (48), but a thorough picture of the distribution of microtubule tyrosination state at the nanoscale level along synapsing axons is still lacking (Box 1). The regulation of the α -tubulin tyrosination cycle is critical for proper neuronal differentiation and outgrowth, and influences axonal transport. Mice lacking tubulin tyrosine ligase (TTL) display disorganization of the cortical layers and die soon after birth (71). The recent identification of vashibins (VASH1 and VASH2) as enzymes catalyzing the removal of the tyrosine residue from α -tubulin (68, 69) opens the door to the interrogation of how the complete tyrosination cycle affects neuronal function and, in particular, axonal transport.

Negatively charged glutamate residues can be sequentially added or removed from the C-terminal chain of α - and β -tubulin by the TTLL (tubulin tyrosine ligase-like) and CCP (cytosolic carboxypeptidase) enzyme families, respectively (72, 73). The graded nature of this modification allows the fine control of several microtubule effectors, including spastin (74) and likely kinesin and dynein motors (see below). Although axonal microtubules are generally highly glutamylated, it is unclear how the graded control of this modification is maintained along the axon and at presynaptic

sites (Box 1). Loss-of-function mutations in the deglutamylase CCP1 affect mitochondrial transport in neurons (75) and cause infantile-onset neurodegeneration (76).

Isoform diversity of tubulin

The differential expression and incorporation of tubulin isoforms remains one of the least understood aspects contributing to microtubule diversity in neurons. Yeast express two α -tubulins and one β -tubulin, *Drosophila* four α - and three or four β -tubulins, *C. elegans* nine α - and six β -tubulins, mice seven α - and seven β -tubulins, and humans eight α - and nine β -tubulins. Members within each tubulin subfamily typically show >90% homology and identical structural properties. Functional specialization of tubulin isoforms has been demonstrated, but only in a few systems such as *Drosophila* axonemes (1). Mutations in tubulin genes can lead to severe neurological disorders, so both the expression pattern of these genes across neuronal populations and the localization of the resulting tubulin isoforms across neuronal compartments are areas of active research (77). The degree of isoform mosaicism in neuronal microtubules is still unknown, and it also remains unclear whether specific tubulin isoforms are preferentially modified posttranslationally, or whether tubulin isoform composition directly affects motor activity and/or MAP recruitment to microtubules. Recently, improved systems for recombinant tubulin expression and isolation have allowed the examination of the impact of specific tubulin isoforms on microtubule dynamics and structure (78). Given the different levels of microtubule dynamicity (9, 49) and length (43) in synaptic and nonsynaptic regions, it is tempting to speculate that microtubules assembled from particular isoforms may be more prevalent in certain neuronal regions, but further studies will be required to investigate these open questions (Box 2).

Regulation of the motors driving axonal transport

The axonal transport of organelles and other cargos is regulated by diverse mechanisms, including modification of the microtubule track through PTMs and/or MAP binding, the biophysical properties of the motor proteins involved, localized activation of motors by adaptors or other binding partners, and the integration of opposing motors bound to the same cargo by associated scaffolding proteins. These mechanisms lead to cargo-specific differences in transport along the axon, so that (for example) mitochondria are trafficked differently from autophagosomes, but also lead to compartment-specific regulation, so that regulation of transport may be differentially tuned in the proximal axon or distal tip. Distinct phases of transport can be identified,

Box 2. Traveling cost of replenishing synaptic vesicles at the presynaptic compartment.

In the human cortex, the total ATP expenditure of a single pyramidal neuron on processes involving ion channel operation during activity and mediating axonal neurotransmission is $\sim 4.8 \times 10^9$ ATP/s (196, 197); 0.92×10^9 ATP/s is consumed to maintain the resting membrane potential (196). Evidence indicates that the energy budget of a single cortical neuron is conserved among humans and rodents at a total of 4.95×10^{14} ATP/day (196). This supply is generated from glycolysis and mitochondrial oxidative phosphorylation (198). Using the mouse basal forebrain cholinergic neuron as a model and considering that SVPs are mostly delivered in the anterograde direction (49), the total distance traveled by SVPs throughout the whole axonal arbor each day is 2.8 to 5.6 km. Because kinesin hydrolyzes one ATP molecule per step, a total of 3.5×10^{11} to 7×10^{11} ATP molecules would be spent in this process (for simplicity we assume a single kinesin-3 dimer mediating the transport of one SVP). This indicates that only 0.07 to 0.14% of the total ATP budget is dedicated to transporting SVPs throughout the axonal arbor of mouse basal forebrain cholinergic neurons. This proportion will likely be higher in rat dopaminergic neurons, which have even larger axonal arbors with $\sim 500,000$ presynapses, or in human serotonergic neurons, which are estimated to extend axons for 350 m (124). Nonetheless, even considering the energy expenditure of axonal SVP transport together with an estimate of ATP spent in the transport of other axonal and dendritic cargos, the ATP directly devoted to microtubule-based transport is likely $<1\%$ of the total ATP budget of a single neuron. This highlights how efficient axonal transport and presynaptic replenishment are from an energetic point of view.

including transport initiation, active transport, and localized delivery (Fig. 3).

In the next sections, we focus on the mechanisms regulating the activity of kinesin-1 and -3 motors (kinesin-1: KIF5A/B/C; kinesin-3: KIF1A) because these are the best-studied motors mediating anterograde cargo transport in the axon. We also discuss the mechanisms regulating cytoplasmic dynein, the sole retrograde motor for axonal transport.

Initiation of transport

Mechanisms regulating transport initiation are critical in allowing entry of kinesin-driven cargos into the axon to sustain cargo supply to presynapses. Mechanisms regulating transport initiation are also critical to govern the motility of dynein-dependent cargo, allowing cargo such as autophagosomes to escape the distal axon and thus clear aging or defective axonal components. Transport initiation mechanisms may also contribute to restarting motility after transient pausing and/or cargo detachment from microtubules, as motors must then bind to an adjacent microtubule to resume transport along the mid-axon.

Free cytosolic kinesin-1 and -3 are locked in an autoinhibited state. Upon binding to cargo, there is a conformational change that unlocks the kinesin motor and allows it to bind strongly to the microtubule. Track engagement initially occurs through an electrostatic interaction between positive charges in the kinesin motor domain and negative charges at the tubulin surface (79) and is stabilized upon ADP/ATP nucleotide exchange in the kinesin motor domain (80). The mode of kinesin-microtubule binding is mostly conserved between kinesin-1 and -3; however, kinesin-3 displays higher affinity for microtubules than kinesin-1 by a factor of ~ 250 (80). The interaction between

the positively charged loop-12 (K-loop) of kinesin-3 and the negatively charged C-terminal tails of α - and β -tubulin contributes to this difference (81–83). Indeed, it is likely that the regulation of the surface charge of the microtubule through the modification of the glutamate side-chain length of the tubulin C-terminal tail (74) can act as a mechanism to modulate kinesin-3 recruitment to the microtubule track along the axon. The nucleotide state of the microtubule can also directly influence motor binding. Kinesin-3 shows lower binding affinity to GTP- than to GDP-like lattices, a feature that is specified by the motor loop-11 region (49). Kinesin-1 was reported to have higher affinity for GTP-like microtubules (56), but others observed this motor binding equally well to GTP- and GDP-like lattices (49, 84).

Motor subunits, accessory proteins, and MAPs can provide another regulatory layer modulating motor-microtubule interaction and transport initiation. For example, kinesin-1 activity is regulated in many contexts by associated kinesin light chains, which contribute to autoinhibition and cargo binding (85). Kinesin-3 is negatively regulated by kinesin-binding protein (KBP), which binds directly to the kinesin-3 motor domain to inhibit motor-microtubule binding (86). KBP does not bind to kinesin-1 motors and thus can act as a selective motor regulator along the axon, although the mechanistic basis for this selectivity remains unclear.

Tau is an axonal MAP that inhibits the binding and motility of kinesin-1 (87, 88) and -3 (88) along microtubules by partially blocking the tubulin surface region with which the kinesin microtubule-binding domain interacts (89). MAP7, on the other hand, promotes microtubule binding of kinesin-1 but inhibits binding of kinesin-3 (88). Detailed studies

have shown that MAP7 interacts with the stalk domain of kinesin-1 (88, 90); this is thought to stabilize a conformation that facilitates binding to the microtubule (90). This effect is important for proper axonal cargo distribution, as the MAP7D2 isoform was recently shown to preferentially localize to microtubules in the proximal axon and regulate kinesin-1 recruitment and axonal sorting of kinesin-1 cargo (97).

Dynein activity is also tightly regulated in the neuron. The dynein complex can exist in two inactive forms, an auto-inhibited “phi (Φ)-particle” (92, 93) and an “open conformation” that does not undergo productive motility (93). To initiate active transport, dynein in the open conformation must bind to dynactin, a multisubunit complex required to activate dynein-driven transport along the axon (94). The binding of an activating adaptor, such as BICD2 or Hook1 (4, 95–97), to form a tripartite dynein-dynactin-activator complex is also required to promote processive motility to the microtubule minus-end. Dynein’s activating adaptors are cargo-specific, acting both to enhance the stability of the dynein-dynactin interaction and to mediate cargo interactions (4). For example, Hook1 activates the dynein-dependent retrograde motility of signaling endosomes but is not required for the motility of mitochondria or autophagosomes (98).

Binding to dynactin promotes the binding of the dynein motor to microtubules by reorienting the dynein dimer to a more optimal binding conformation (96) and by mediating a direct interaction with microtubules through its glycine-rich (CAP-Gly) domain (99). The affinity of dynactin for microtubules is directly regulated by tubulin tyrosination (48, 100), a factor that spatially specifies the initiation of dynein-driven transport along the microtubule lattice. Dynein is robustly recruited to the dynamic plus-end of microtubules as part of a molecular program that involves the sequential recruitment of EB and CLIP-170 proteins (48, 101). This mechanism is critical for dynein-based transport initiation at distal regions of the axon (48, 101) but might also play a role at presynaptic sites, given the high dynamicity of the local microtubule network (49).

Active transport

Once transport is initiated, sustained motor activity conveys cargo along the axon, mediating cargo distribution to appropriate destinations such as presynapses or the axon terminal. The intrinsic biophysical properties of the motor are a major determinant of how far a cargo is transported prior to detachment from the microtubule. Kinesin-1 motors take 8-nm steps, which corresponds to the repeating $\alpha\beta$ -tubulin dimer unit in the microtubule lattice. The two heads of kinesin-1 step alternately in a hand-over-hand fashion to produce processive motility. In vitro, kinesin-1 can take

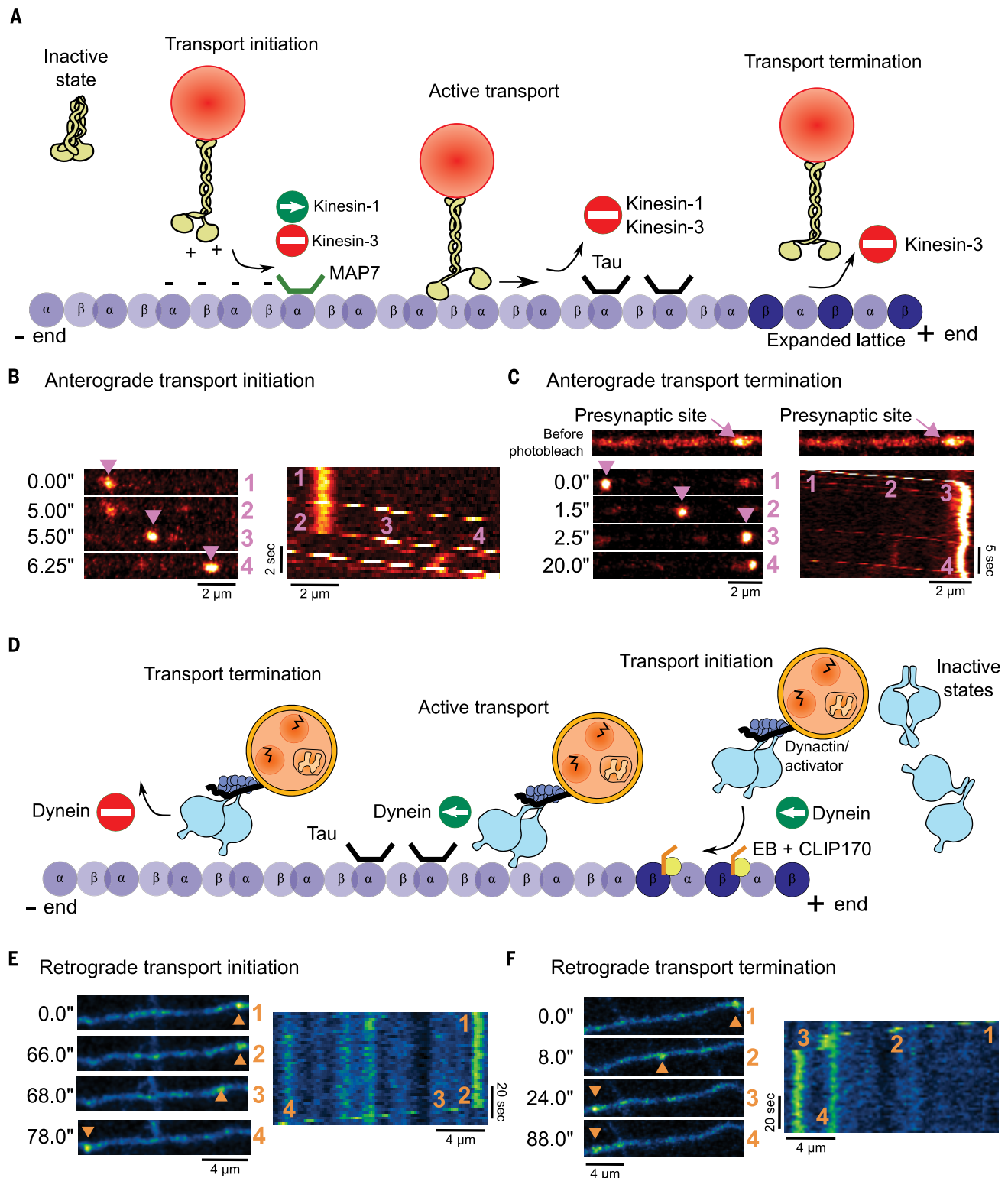


Fig. 3. Axonal transport is regulated at multiple levels. (A and D) Despite having distinct biophysical properties, kinesin (A) and dynein (D) undergo similar transport phases. From an inactive unbound state, the motors bind to cargo and are recruited to the microtubule where they initiate active transport. MAPs, such as Tau or MAP7, can differentially affect motors and induce transport termination in a motor-specific manner. Kinesin-3 can sense the nucleotide state of the microtubule lattice and rapidly detaches upon encountering a dynamic GTP microtubule plus-end. (B and C) Initiation (B) and termination (C) of

synaptic vesicle precursor (SVP) transport along an axon of a live hippocampal neuron in culture expressing synaptophysin-mScarlet. SVPs are transported mostly in the anterograde direction by kinesin-3 and pause preferentially at presynaptic sites, which are enriched in dynamic GTP-rich microtubule plus-ends. (E and F) Initiation (E) and termination (F) of autophagosome transport along the axon of a live cultured hippocampal neuron expressing LC3-GFP. Autophagosomes are mostly generated distally in the axon and driven by dynein in the retrograde direction toward the soma.

more than 100 steps prior to detachment, totaling a distance of ~1 μm , whereas dimeric kinesin-3 can take more than 500 steps and cover a distance of ~8 μm in a single run (49). In contrast to kinesin, dynein step size can be variable (8 to 32 nm) (102). The binding of dynein to dynactin and an activating adaptor such as BICD2 or Hook1 (4, 95, 103) modulates motor processivity and velocity, resulting in run lengths of 5 to 10 μm along a single microtubule and velocities of 0.8 to 1.3 $\mu\text{m/s}$ (104). Mechanistically, longer run lengths and faster velocities can be explained by the capacity of certain activating adaptors, including BICD2 and Hook3, to recruit two dynein motors to a single dynactin complex (104, 105).

In vivo, the biophysical properties of motor proteins are tightly regulated by the formation of high-order motor assemblies or by interaction with accessory proteins. Motors can team up in homo- (106) or heteromeric (107) assemblies to drive organelle transport. Non-invasive force measurements have determined that synaptic vesicle precursors in *C. elegans* neurons can be transported by one to four active kinesin-3 dimers (106). Estimates of motor number on axonal cargos such as late endosomes/lysosomes range from one to four kinesin motors and one to five dynein motors (50). Measurements of motor number using quantitative super-resolution microscopy confirm these estimates (108). Cooperation affects dynein and kinesin function differentially. Whereas dynein teams optimize force production (109), multiple kinesin-1 motors bound to a single cargo mostly affect microtubule binding rates (110, 111).

Most organelle cargos examined to date, including late endosomes/lysosomes, mitochondria, and autophagosomes, generally have oppositely directed dynein and kinesin motors bound simultaneously (112). The coordination between opposing motors can be governed by scaffolding proteins that mediate organelle-motor interaction (112). JIP1 is a scaffolding protein that regulates the trafficking of both autophagosomes and APP along the axon by binding opposing dynein and kinesin motors and selectively regulating their activity (113, 114). Similarly, TRAKs 1 and 2 are scaffolding proteins associated with mitochondria that are also thought to selectively regulate bound dynein and kinesin motors to control mitochondrial motility along the axon (115). Together, adaptors and scaffolding proteins provide cargo-specific regulation of organelle trafficking along the axon (4, 112). Motor activity can be further tuned by modulations of the phosphorylation state of the motors or their associated adaptors and scaffolding proteins (116).

Recent evidence also implicates MAPs in motor regulation. The inhibitory effect of Tau on kinesin motility (87) was shown to preferentially induce dynein-mediated minus-end-

directed transport of vesicles (117). Contrastingly, by promoting kinesin-1 recruitment and motility (88, 90, 118), decoration of microtubules with MAP7 biased vesicle transport to the plus-end (118). Given that MAP7 also exerts an inhibitory effect on kinesin-3 (88), these studies raise the interesting possibility that certain microtubules may be selectively decorated with MAPs to act as highways for specific motor or organelle populations, or alternatively to favor transport in a specific direction.

Transport, distribution, and delivery of axonal cargo

Synaptic vesicle precursors

Synaptic vesicle precursors (SVPs) are vesicles enriched in synaptic components such as synaptophysin, synaptotagmin, and vesicular amino acid transporters (e.g., VGLUT1) that replenish synaptic vesicle and active zone components at presynaptic sites. SVP transport is mainly driven by kinesin-3 (119), but kinesin-1-mediated transport of vesicles carrying active zone components has been reported (120). SVP movement along the axon is highly processive and efficient, averaging >3 $\mu\text{m/s}$ with few pauses (49); these pauses occur preferentially at microtubule termini (45). Presynaptic delivery of SVPs is specified by the high local density of GTP-rich dynamic microtubule plus-ends and the intrinsic low affinity that kinesin-3 displays for GTP-rich microtubule lattices (49). DENN/MADD (121) and Arl-8 (122, 123) have also been implicated in the regulation of SVP presynaptic delivery. By binding the small guanosine triphosphatase (GTPase) Rab3 and the kinesin-3 stalk domain, DENN/MADD promotes binding of the SVP to the microtubule (121). Because DENN/MADD binds GDP-Rab3 less efficiently, the nucleotide state of Rab3 can regulate SVP association with kinesin-3. Similarly, the small GTPase Arl-8 has been shown to bind and activate kinesin-3 when GTP-bound, but in the GDP state the binding is minimal (123). This GTPase nucleotide switch may be an important step to prevent SVP from escaping the presynapse by disengaging the SVP from the motor or preventing the SVP-motor complex from resuming transport after detaching from the microtubule.

The scale of successfully maintaining presynaptic site homeostasis and replenishing SVPs in a timely and spatially precise manner is becoming increasingly clear with advances in axonal tracing techniques, transport studies, and more accurate determination of protein lifetimes in neurons. For instance, the axonal arbor length of a cholinergic neuron projecting from the mouse basal forebrain is on average 30 cm and can branch >1000 times (124). Assuming the presence of a presynapse at each branch point and an average intersynaptic distance of 4 μm along the entire axon, a simple model can be built in which a 4000- μm pri-

mary axon branches 296- μm secondary axons 1000 times. This results in 75 presynapses per branch and 75,000 total distributed throughout the entire axonal arbor. Presynaptic boutons contain ~200 synaptic vesicles (125). Studies in cultured neurons estimate that the average presynaptic protein lifetime is ~3.5 days (126) and that half of the synaptic vesicles residing at presynapses are turned over in <30 hours (127). Presynaptic protein lifetimes are 2 to 4 times longer in vivo (128). Using this range to scale up synaptic vesicle lifetime, we can estimate that presynaptic sites are replenished with 20 to 40 new SVPs per day. This totals 1.5 to 3 million SVPs traveling a total distance of 2.8 to 5.6 km along the axon to replenish presynapses every day, and a staggering 17 to 35 SVPs being generated each second in the cell body (Box 2). Early studies have determined that the exit flux from the Golgi in cell lines can reach 4000 proteins per second (129). Given that a synaptic vesicle is composed of ~250 proteins (130), 16 synaptic vesicles could be produced at that rate. Considering that numerous other vesicles of the secretory pathway (e.g., lysosomes, postsynaptic cargos) need to be simultaneously generated, one can contemplate the astonishingly high metabolic and secretory capacity of the ER and Golgi complex in neurons. Because synaptic vesicle lifetime is inversely proportional to the number of exocytosis-endocytosis cycles it undergoes (127), it is likely that the replenishment rate of synaptic vesicles is modulated across neuronal populations with distinct firing patterns.

Mitochondria

A large part of the metabolic capacity of the neuron derives from mitochondria. The proper positioning of these organelles along the axon is critical for synaptic function. Mitochondrial movement is mainly regulated by kinesin-1 and dynein (115). In peripheral axons with terminal synapses, mitochondria tend to move processively in a single direction with an anterograde bias (131). In CNS axons, mitochondrial motility decreases in parallel with age and synaptic connectivity (132, 133). About half of mitochondria in the axon are stationed at presynaptic sites (133, 134), where they are an important ATP source (135) and regulate Ca^{2+} levels (134, 136). Delivery of mitochondria to presynaptic sites is controlled by local Ca^{2+} influx, which causes the accessory protein Miro on the mitochondrial outer membrane to change conformation and inhibit kinesin-mediated transport (137, 138). The acetylation state of Miro, which is in part controlled by the deacetylase HDAC6, inversely affects the sensitivity of Miro to Ca^{2+} (139). Because both kinesin and dynein interact with Miro via TRAK proteins (115), it is likely that this mechanism affects dynein-mediated transport as well. Despite the transient nature of Ca^{2+}

influx at presynapses, mitochondria can remain stationed at these sites for days (133). The mechanisms controlling the long-term anchoring of mitochondria to the presynapse are still unclear, but there is evidence suggesting that syntaphilin docks mitochondria to presynaptic microtubules (140). Local myosin-actin interactions (141) and stable mitochondria-ER or mitochondria-plasma membrane contacts might also play a role.

Dense-core vesicles

Dense-core vesicles (DCVs) convey a wide variety of neuropeptides along axons in a neuron type-dependent manner (142). DCV transport is mainly mediated by kinesin-3 (143) and dynein (144), although kinesin-1 might also be involved (145). Anterograde-moving DCVs frequently pause at presynaptic sites (49), where they are preferentially retained in an activity-dependent manner (49, 146, 147).

Late endosomes and lysosomes

The anterograde transport of LAMP-1-positive vesicles (late endosomes and lysosomes) (148, 149) in axons is mainly mediated by kinesin-1 through an association with the BORC-Arl-8-SKIP complex (150), although kinesin-2 might also play a role (50). Dynein drives the retrograde motility of these organelles, regulated by JIP3 (151) and RILP in conjunction with Rab7 (152, 153). LAMP-1-positive vesicles move as rapidly as SVPs but pause more frequently (49). These pauses occur randomly along the axon, unlike the preferential pausing at synapses observed for SVPs (49), and likely underpin the even distribution of LAMP-1 vesicles observed throughout the axonal compartment (150). Late endosomes and lysosomes are involved in many processes; they are critical players in neuronal degradative pathways (154) and Ca^{2+} regulation (155). Recently, late endosomes (Rab7- and LAMP-1-positive vesicles) were observed to associate with RNA granules and to serve as platforms for local translation along the axon (156). Lysosomal dysfunction particularly affects presynaptic maintenance and homeostasis (157). An even distribution of these vesicles maintained by a random pausing pattern might be optimal to ensure efficient fusion with autophagosomes and sustain protein synthesis along the whole axonal compartment. Nonetheless, given the heterogeneous nature of these organelles (50, 148), a more thorough analysis of their transport and distribution using multiple markers simultaneously might reveal a more biased distribution of certain subpopulations to specific axonal sites and uncover their specialized roles.

Autophagosomes

Autophagosomes and signaling endosomes are two vesicle populations characterized by

robust retrograde transport throughout the axon. Both vesicle populations are generated distally in the axon (154, 158, 159), where dynein (160) and dynactin (101) are enriched. Autophagosomes initially show bidirectional oscillatory movement before engaging in processive retrograde transport (154). The accessory protein JIP1 was shown to be critical in resolving the tug-of-war between opposing motors by inhibiting kinesin-1 and allowing unopposed dynein-based movement (114). Huntingtin and HAP1 are also implicated in the regulation of autophagosome-bound motors (161). Robust retrograde transport of autophagosomes is intermittently interrupted along the axon; these pauses may correspond to fusion events with late endosomes and lysosomes. Ultimately, axonal autophagosomes and autophagolysosomes are conveyed to the soma for the final steps in the degradation of engulfed proteins and organelles and the recycling of their components.

Signaling endosomes

Dynein activity is also crucial for signaling endosome formation and processive transport to the cell body (98); both in transit and upon arrival in the soma, these organelles regulate signaling pathways affecting neuronal survival, neuronal development, and synapse formation (159). The motility of signaling endosomes is regulated by the dynein adaptors Hook1 (98) and RILP (153). Given the diversity of signaling endosomes (162), research will be required to elucidate whether distinct populations use different adaptors or activators depending on their maturation state, or whether specific dynein adaptors or activators bind to particular receptor-ligand duos.

Cytosolic cargos

Soluble protein supply to the axon can be sustained by slow axonal transport of proteins generated in the soma (5). Slow axonal transport (0.2 to 10 mm/day) has been directly shown for cytoskeleton elements, including tubulin, actin, and dynein (5, 160, 163), and also for some presynaptic components, such as synapsin (164). Except for actin (163), slow axonal transport is largely dependent on microtubule-based fast transport and, in particular, kinesin-1 movement through the transient association of the soluble cargo with the motor (160, 164). It is unknown to what extent neurons rely on slow axonal transport to supply soluble proteins to presynaptic sites. However, it is clear that the sole reliance on this mechanism would severely limit the capacity of a human central cholinergic neuron to rapidly adjust the supply of soluble protein throughout its entire >100-m axonal arbor (124) upon sudden surges in local protein demand. Moreover, a soluble protein synthesized in the soma would take more than 100 days to reach the tip of a 1-m human lower motor neuron axon, 450 days to the tip of the 4.5-m recurrent laryngeal nerve axon in the giraffe, and 8 years to the tip of a 30-m spinal axon in blue whales. These numbers are incompatible with the lifetime of most cytosolic proteins in neurons (half-life of tubulin, ~30 days; of dynein heavy chain, ~6 days) (128).

RNA transport

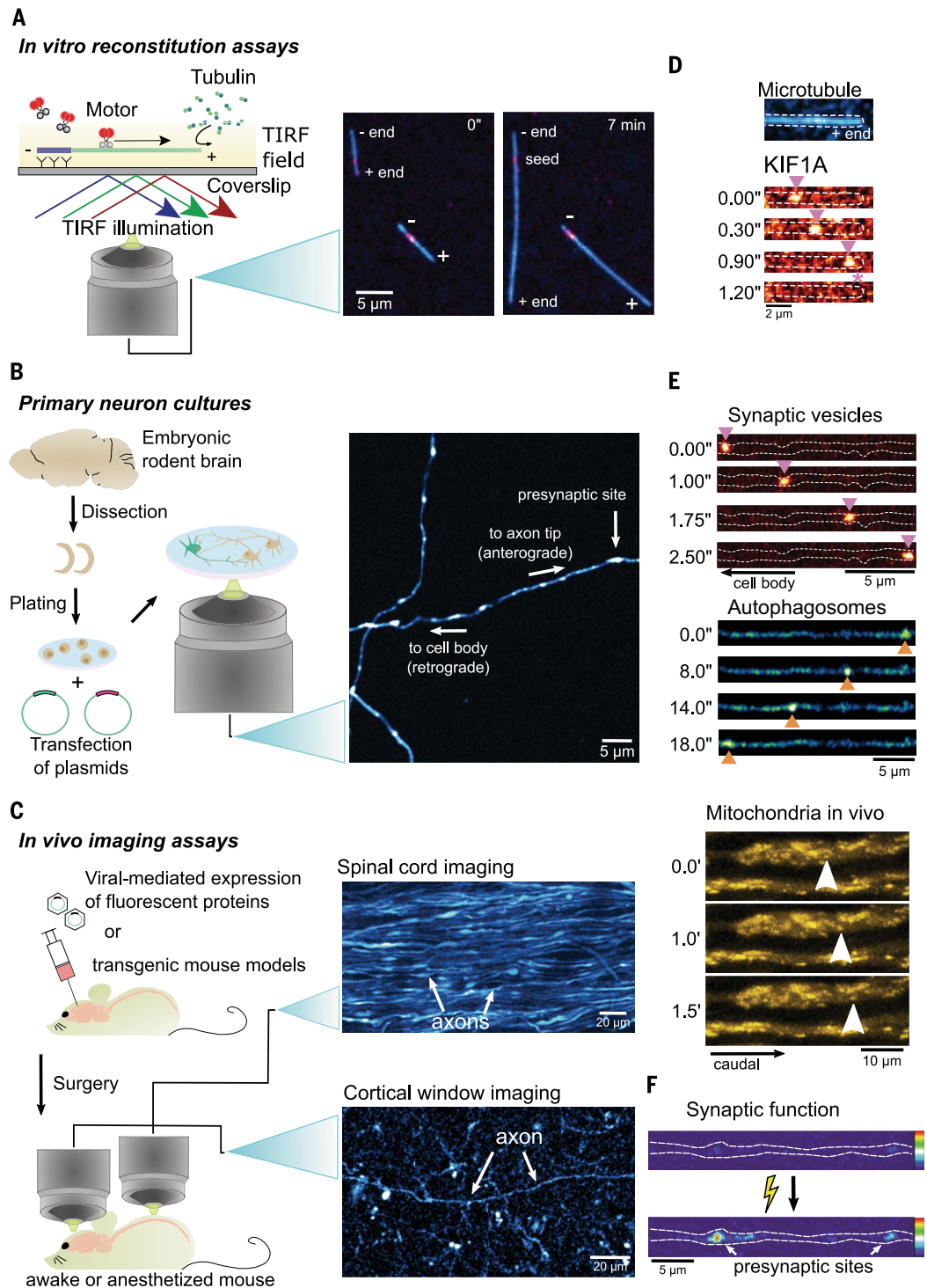
Axons are populated by a wide range of mRNA transcripts (165), which are actively translated in vivo (166). Local translation of mRNAs in the axon circumvents the limitations related with short protein lifetimes and, in principle,

Box 3. Outstanding questions.

Among the many open questions in neuronal cell biology, one of the most fundamental concerns how the subcompartmentalization of the neuron is specified. An increasing number of molecular players involved in the stabilization of the microtubule network at the AIS have been described, but the exact mechanisms that sort these into the neurite that is to become the axon, and/or exclude them from pre-dendritic processes, are still unclear. There are at least 20 kinesin motor isoforms expressed in hippocampal neurons (199). The function of most of these motors in neurons and their relevance for axonal transport are still unclear. Furthermore, the roles of dynein-activating adaptors in defining dynein cargo recognition and transport regulation in neurons will also be an active area of research. In addition, a detailed depiction of the microtubule network organization along whole axons is still absent. Improved cryo-EM techniques and computational approaches to reconstruct serial sections will play an important role in unveiling this. Correlative light and electron microscopy approaches will assist in deepening our understanding of microtubule dynamics and organization. A particular area of interest will be to unambiguously demonstrate that incorporation of native GTP-tubulin into a preexisting microtubule lattice occurs in vivo. Local translation in the axon potentially has important implications for the replenishment of soluble proteins and maintaining a healthy pool of mitochondria at presynaptic sites. The mechanisms that mediate the distribution and stability of mRNA and the transport and/or assembly of ribosomes throughout the axonal arbor are not well understood and constitute an area of interest for future studies. Finally, the growing list of known mutations affecting components of the axonal transport machinery and causative of a broad range of neurological disorders highlights gene-editing approaches as a promising research avenue to uncover suitable therapeutic strategies for these disorders.

Fig. 4. Range of imaging approaches to address questions in the field of axonal transport.

A wide range of imaging assays can be used to investigate the dynamics of biological processes in systems with different levels of biological complexity. Examples: (A) Combining in vitro reconstitution assays with total internal reflection fluorescence (TIRF) microscopy allows the assessment of the dynamic behavior of single motors on microtubules. The ability to easily manipulate the system by adding or removing components enables the elucidation of how single factors (e.g., MAPs) affect motor activity or what minimum components are required in the system to observe a specific dynamic behavior. Panels show the basic setup of an in vitro reconstitution assay and TIRF microscopy imaging of polymerization of fluorescently labeled tubulin. (B) Primary rodent CNS neurons in culture undergo a well-described polarized developmental process akin to what is observed in vivo. Neurons develop an axon and multiple dendrites, establish synaptic contacts with their neighbors, and fire action potentials spontaneously. The combined ability to easily manipulate the neurons genetically and pharmacologically, express fluorescent reporters, and use imaging techniques with high spatial and temporal resolution (such as spinning-disk confocal microscopy) makes this system ideal to investigate the mechanisms underlying organelle dynamics in a physiologically relevant context. Panels show a basic protocol to image primary neuron cultures and a confocal microscopy image of hippocampal axons in culture expressing fluorescently tagged synaptophysin. (C) The development of Cre mouse lines, improved adeno-associated viral vectors, and the advent of CRISPR/Cas9 gene-editing techniques have enabled more straightforward strategies of genetically manipulating mice, generating transgenic mouse lines, and expressing fluorescent reporters even in a neuron subtype-specific manner. Innovative surgical techniques, including spinal cord exposure and cortical window implementation, allow long-term optical access to the spinal cord and superficial layers of the cortex. By using two-photon microscopy techniques, it is thus possible to interrogate organelle dynamics and synaptic function in neurons in their native environment. Panels show a simple strategy to perform in vivo imaging assays, an image of axonal bundles in the spinal cord of a Thy1-YFP mouse, and a cholinergic axon in cortical layer I/II of a ChAT-Cre ROSA26-EGFP^f mouse. (D) TIRF imaging of a single dimeric KIF1A motor labeled with tetramethylrhodamine actively moving on a fluorescently labeled microtubule in an in vitro reconstitution assay. (E) Two-photon microscopy imaging of mitochondrial dynamics in spinal cord axons of a Thy1-MitoCFP mouse. Synaptic vesicle precursors (labeled with synaptophysin-mScarlet) move mostly in the anterograde direction, whereas autophagosomes (labeled with LC3-GFP) show robust retrograde movement along the axons of live neurons in culture. (F) VGLUT1-pHluorin signal in a primary hippocampal neuron before and after electrical-field stimulation. An increased repertoire of fluorescent pH and Ca²⁺ indicators are allowing the study of how a variety of processes and mechanisms regulating axonal transport affect synaptic function and neuronal activity.



can rapidly respond to changes in protein demand and tune local protein supply. However, this mechanism is likely limited to expression of cytosolic proteins because there is limited Golgi in the axon, at least in rodents (158). It is possible that in larger mammals, there is a higher prevalence of Golgi in the axon to locally synthesize membrane-associated proteins rather than waiting days for export from the soma. In neurons, RNA-binding proteins bind to mRNA, forming specialized granules with liquid-like properties (167), and evidence suggests that their transport can be directly mediated by kinesin-1 and dynein (168, 169). Recently, it was shown that axonal mRNA distribution can also be achieved by hitchhiking on late endosomes (156). The mechanisms controlling mRNA stability or the distribution of ribosomes necessary for local translation along the axon remain unclear. Future studies are needed to address these questions and reveal the key roles of local translation in axonal and presynaptic function (Box 3).

Axonal transport and disease

From motor proteins to adaptors to tubulin subunits, mutations affecting components of the axonal transport machinery have been implicated in a broad range of neurological disorders. Advances in DNA sequencing techniques have identified mutations in kinesin-1 and kinesin-3 as causative of a broad range of neurological disorders, including hereditary spastic paraplegia, amyotrophic lateral sclerosis, epilepsy, and severe intellectual deficits (170–173). Similarly, mutations in dynein, primarily in the *DYNC1H1* gene encoding the cytoplasmic dynein heavy chain, have been implicated in a range of neurodevelopmental and neurodegenerative diseases [reviewed in (174)], including Charcot-Marie-Tooth disease type 2O (CMT-2O) and spinal muscular atrophy–lower extremity predominant. Mutations in kinesin- and dynein-binding proteins have also been implicated in disease. For example, missense mutations on the kinesin-3 inhibitor KBP were found to be causative of Goldberg-Shprintzen syndrome (175), a severe neurological disorder characterized by microcephaly and mental retardation. Mutations in the dynein-binding protein Lis1 cause severe lissencephaly, primarily due to defects in neuronal migration, but recent work has also established a critical role for Lis1 in maintaining axonal transport in the adult brain (176). Mutations in the *DC1N1* gene encoding the p150^{Glued} subunit of dynactin affect the initiation of retrograde transport (101); these mutations are directly implicated in Perry syndrome, an aggressive form of Parkinsonism (177). A distinct mutation within the same domain causes a rare form of motor neuron disease, HMN7B (178). Similarly, mutations in *BICD2*, which encodes an activating adaptor for dynein, lead

to spinal muscular atrophy (179–181) characterized by lower motor neuron degeneration.

Mutations in associated effectors or scaffolding proteins can also lead to disease. For example, Rab7 is anchored to the membrane of late endosomes and lysosomes and is involved in dynein/dynactin motor complex recruitment (152). Mutations in Rab7 have been implicated in Charcot-Marie-Tooth disease (182) and shown to disrupt endosome axonal transport in sensory neurons (183). Huntingtin can act as a motor scaffold coordinating the activity of kinesin/dynein motor complexes (112). Pathogenic expansion of the polyQ repeat region of huntingtin deleteriously affects the scaffolding functions of the protein, disrupting the axonal transport of multiple cargos, including autophagosomes and mitochondria (161, 184, 185). Finally, mutations that alter microtubule organization or regulation can cause disease. For example, mutations in various α - and β -tubulin isoforms lead to brain malformations and neurodevelopmental disorders (186). Two have been located at or near the GTP nucleotide-binding pocket of β -tubulin (187, 188) and likely affect the nucleotide state transition directly, whereas one in α -tubulin was recently shown to directly impair dynein motor activity (189).

Axonal dynamics are a tightly orchestrated mechanism required to maintain neuronal health and function through a lifespan that may reach 90 to 100 years, so it is not surprising that mutations that disrupt these dynamics are tightly associated with both neurodevelopmental and neurodegenerative disease. However, it remains unclear why mutations in genes required for axonal transport result in such a wide variety of diseases, affecting very different stages of development or distinct neuronal subtypes. For example, mutations within the *DYNC1H1* gene encoding the cytoplasmic dynein heavy chain can induce either intellectual disability or distal limb weakness, or both (174). As we learn more about the specific interactions among motors, adaptors, and cargo being transported, the pathobiology induced by mutations in the axonal transport machinery may become clearer.

Directly targeting the transport machinery and the microtubule network to restore rates of axonal transport has long been considered a promising therapeutic strategy to slow the progression of neurodegenerative diseases. More recently, the pharmacological modulation of microtubule dynamics with microtubule-stabilizing drugs has been shown to promote axonal regeneration after axonal injury (190–192). It is known that axonal transport plays a critical role in sustaining axonal regeneration (193); whether the pro-regeneration effects of microtubule-stabilizing drugs are directly associated with a boosting of anterograde and retrograde trafficking to and from the injury site remains to be elucidated.

Concluding remarks

Axonal transport sustains synaptic and neuronal function by delivering the necessary components to support neurotransmission at presynaptic sites and carrying aged organelles or signaling vesicles to the soma. From the functional diversity provided by tubulin isoforms and PTMs to the different ways motors engage with microtubules or interact with accessory proteins to bind cargo, the distinct layers that regulate axonal transport are many but coalesce to efficiently direct the distribution of a wide range of cargo along the axonal arbor. Recent advances in single-molecule and in vivo approaches are not only enabling a deeper understanding of how these regulatory layers interplay; they are also uncovering the true scale of the task that maintains hundreds of thousands of synapses operating effectively within the same neuron. Further application of innovative and complementary approaches will continue to build a coherent view of the mechanisms that maintain axonal and synaptic homeostasis through life (Fig. 4).

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RESEARCH ARTICLE SUMMARY

NEUROSCIENCE

The forebrain synaptic transcriptome is organized by clocks but its proteome is driven by sleep

Sara B. Noya, David Colameo, Franziska Brüning, Andrea Spinnler, Dennis Mircsof, Lennart Opitz, Matthias Mann, Shiva K. Tyagarajan*, Maria S. Robles*, Steven A. Brown*

INTRODUCTION: Temporally consolidated behaviors such as sleep normally occur in synchrony with endogenous circadian rhythms and both have been reported to contribute to global daily oscillations of transcription in brain. Neurons have further adapted specialized means to traffic RNA into distant dendritic and axonal arbors, where it is locally translated. Together, these mechanisms allow coordination of physiology with environmental needs.

RATIONALE: About 6% of the forebrain transcriptome oscillates in a time-of-day-dependent manner, and it has been proposed that this oscillation is mostly driven by the sleep-wake state to enable daily changes in synaptic structure and function. In turn, such synaptic scaling is thought to form a critical feature of the sleep-wake process. Given the highly local capacity for synaptic remodeling, an essential missing link in this argument is the effects of circadian clocks and sleep pressure upon messenger RNA (mRNA) and related protein abundance at synapses themselves. To address this question, we examined daily rhythms of transcript and

protein abundance in transcriptome and proteome of synapses from the mouse forebrain, using biochemically purified synaptoneuroosomes isolated across the 24-hour day both at normal sleep pressure and at constant high sleep pressure.

RESULTS: Notably, 67% of synaptic mRNAs showed circadian oscillations, with a mean amplitude of about twofold. Further, 93% of these oscillating transcripts were exclusively rhythmic in synaptoneuroosomes, suggesting an entirely posttranscriptional origin for synaptic mRNA oscillations. This observation was supported by single-molecule fluorescence in situ hybridization. Rhythmic synaptic transcripts formed two distinct waves, anticipating either dawn or dusk, and both required a functional circadian clock. These two waves showed completely different functional signatures: synaptic signaling preceded the active phase, whereas metabolism and translation preceded the resting phase. Comprehensive circadian characterization of the synaptic proteome demonstrated the functional relevance

of this temporal gating for synaptic function and energy homeostasis. Overall, the oscillations of 75% of synaptic proteins were concomitant with their rhythmic transcripts, indicating a key role for local synaptic translation. Under conditions of high sleep pressure, one-fourth of mRNAs remained identically circadian, and most preserved some degree of circadian rhythmicity. In contrast, no substantial circadian rhythm could be detected

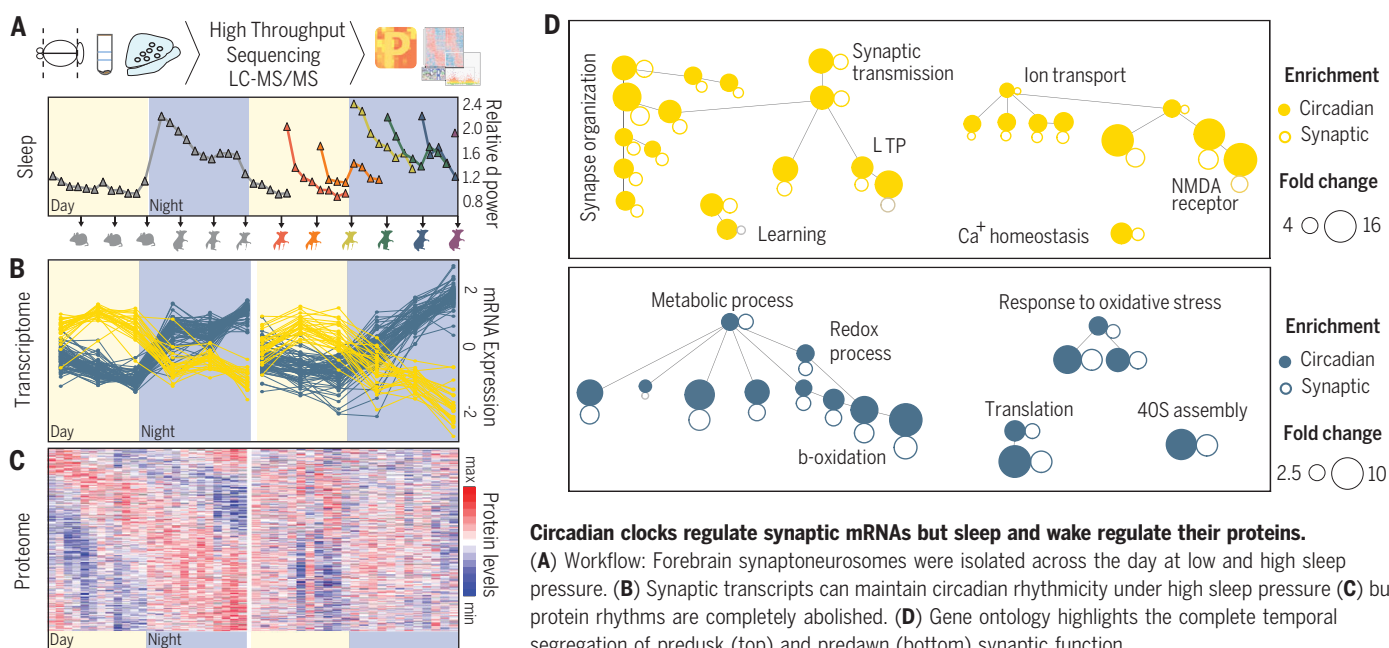
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in any protein when sleep pressure was constantly high.

CONCLUSION: Examining the dynamics of mRNAs in the synaptic landscape revealed the largest proportion of circadian transcripts in any tissue, cell, or organelle described to date. These synaptic oscillations are controlled posttranscriptionally and the daily dynamics of transcripts and their related proteins clearly delineate different cellular modes between sleep and wake. Our study provides insight into the connectivity between sleep and circadian rhythms and suggests an elegant paradigm whereby a molecular clock provisions synapses with mRNAs before dawn and dusk, which are later translated in response to activity-rest cycles. ■

*Corresponding author. Email: steven.brown@pharma.uzh.ch (S.A.B.); charo.robles@med.uni-muenchen.de (M.S.R.); tyagarajan@pharma.uzh.ch (S.T.)
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Circadian clocks regulate synaptic mRNAs but sleep and wake regulate their proteins.

(A) Workflow: Forebrain synaptoneuroosomes were isolated across the day at low and high sleep pressure. (B) Synaptic transcripts can maintain circadian rhythmicity under high sleep pressure (C) but protein rhythms are completely abolished. (D) Gene ontology highlights the complete temporal segregation of predusk (top) and predawn (bottom) synaptic function.

RESEARCH ARTICLE

NEUROSCIENCE

The forebrain synaptic transcriptome is organized by clocks but its proteome is driven by sleep

Sara B. Noya¹, David Colameo^{1*}, Franziska Brüning^{2,4}, Andrea Spinnler¹, Dennis Mircsof¹, Lennart Opitz³, Matthias Mann^{4,5}, Shiva K. Tyagarajan^{1†}, Maria S. Robles^{2†}, Steven A. Brown^{1†}

Neurons have adapted mechanisms to traffic RNA and protein into distant dendritic and axonal arbors. Taking a biochemical approach, we reveal that forebrain synaptic transcript accumulation shows overwhelmingly daily rhythms, with two-thirds of synaptic transcripts showing time-of-day-dependent abundance independent of oscillations in the soma. These transcripts formed two sharp temporal and functional clusters, with transcripts preceding dawn related to metabolism and translation and those anticipating dusk related to synaptic transmission. Characterization of the synaptic proteome around the clock demonstrates the functional relevance of temporal gating for synaptic processes and energy homeostasis. Unexpectedly, sleep deprivation completely abolished proteome but not transcript oscillations. Altogether, the emerging picture is one of a circadian anticipation of messenger RNA needs in the synapse followed by translation as demanded by sleep-wake cycles.

A cell-autonomous circadian clock based upon feedback loops of transcription and translation functions in nearly every cell of the mammalian body and influences most aspects of physiology. In the brain, the most obvious manifestation of circadian control is the consolidation of sleep into day or night. In synchrony with sleep and wake, daily rhythmic oscillations also occur in a substantial fraction of the brain transcriptome, ranging from 3 to 4% in retina, cerebellum, brainstem, and hypothalamus, to 8 to 10% in cortex and suprachiasmatic nuclei (1–4). These oscillations are likely driven in part by rhythmic assembly and disassembly of transcription complexes and chromatin modifiers orchestrated by circadian clock-specific activators and repressors such as BMAL1/NPAS1, PERIOD (PER1 and PER2), and CRYPTOCHROME (CRY1 and CRY2), as observed in other tissues (5–8). However, it has become increasingly apparent that posttranscriptional processes play an important role in circadian regulation. In liver and suprachiasmatic nuclei (SCN), the best-studied circadian tissues to date, evidence of circadian

mRNA processing, polyadenylation, and translation have appeared (9–11). Complicating matters still further, circadian oscillations in mRNA levels can also be driven by temporally consolidated behaviors. In liver, timing of food intake contributes to rhythmic transcription (12), and in cortex, sleep-wake cycles play a major role (3).

Relative to all other cell types, neurons present a special case because mRNA is distributed to potentially distant compartments such as axonal terminals and postsynaptic spines. Although the axodendritic arbor contains only ~10% of total transcripts (13), a large literature demonstrates that mRNAs are actively transported to neurites (14–16). Downstream, local synaptic translation of mRNA has been postulated to be an important mechanism in memory (17, 18), and both synaptic size and total protein abundance are dynamically scaled by wake and sleep (19, 20).

Despite this context, circadian and sleep-wake-dependent regulation of synaptic transcript pools in brain, as well as their functional importance, remain entirely unexplored questions. “Multi-omic” approaches have been increasingly used to understand complex questions of cellular regulation, and therefore might also be useful to explore the connectivity between the clockwork and sleep (21). Here, we used a combination of biochemical fractionation, deep sequencing, single-transcript confocal microscopy, and mass spectrometry (MS)-based quantitative proteomics to analyze the origin and function of rhythmic daily oscillations in the transcripts and proteins of forebrain synapses. From these studies, we derive the simple paradigm that a clock-gene-dependent mechanism is needed to provision synapses with mRNAs in

a circadian fashion, which are then translated in response to sleep-wake cycles.

The forebrain synaptic transcriptome shows pervasive daily rhythmicity

To generate a time-resolved map of the synaptic transcriptome from the mouse forebrain, we purified synaptoneurosomes using biochemical fractionation with discontinuous Percoll gradients (22). Synaptoneurosomes represent axonal nerve terminals (cytoplasm, synaptic vesicles, mitochondria, and cytoskeleton) and attached postsynaptic structures. We collected forebrains every 4 hours across the day in biological triplicates under natural conditions of light and dark (LD) and identified mRNAs by high-throughput sequencing in the forebrain homogenates and the purified synaptoneurosomes (Fig. 1A). After normalization and thresholding (see materials and methods), we identified 14,073 unique transcripts. These transcripts overlapped almost completely with those identified in the hippocampal neuropil (23), a highly projection-enriched brain region (Fig. 1B), as well as with other similar transcriptomes of forebrain synapses (24) (fig. S1A). In addition, quantitative polymerase chain reaction demonstrated enrichment of known synaptic mRNAs and depletion of nuclear ones (fig. S1B). These measures confirmed the validity of our biochemical approach. Reasoning that a fold change of ≥ 1.5 in synaptic versus forebrain abundance should represent a reasonable criterion with which to identify a specific synaptic element, we found 3104 unique synaptically enriched mRNAs (Fig. 1B and table S1). Gene Ontology (GO) analysis corroborated the overwhelming predominance of synaptic annotations among these transcripts, as well as their stepwise enrichment across the analytical steps of the procedure (Fig. 1C).

Using the Perseus computational platform (25), we analyzed these synaptically enriched mRNAs for evidence of daily rhythmicity (period 24 hours, $q < 0.05$), and found that 2085 (67%) were cycling, the highest proportion of rhythmic transcripts estimated in any tissue, cell, or organelle described to date (Fig. 1D and table S2; results with other q -value cutoffs are shown in fig. S1C). The average fold change was 1.8 (Fig. 1E) and the majority showed a peak-trough amplitude of 1.5 or greater (fig. S1D). The 2085 cycling features overlapped substantially with those detected using other algorithms (91% with the JTK_CYCLE algorithm; period 24 hours, $q < 0.05$) (26) (fig. S2A). In parallel, we analyzed the forebrain transcriptome and found that only 6% of it was oscillating (Fig. 1F, fig. S2B, and table S3). Notably, 93% of synaptic circadian transcripts were cycling exclusively in the synapse; only 7% were cycling also in the whole forebrain, and these with reduced amplitudes (Fig. 1G and fig. S2C). This minimal overlap implies that daily oscillations

¹Institute of Pharmacology and Toxicology, University of Zürich, Zürich, Switzerland. ²Institute of Medical Psychology, Medical Faculty, LMU Munich, Germany. ³Functional Genomics Center Zurich, University of Zurich–Eidgenössische Technische Hochschule, Zurich, Switzerland. ⁴Department of Proteomics and Signal Transduction, Max Planck Institute of Biochemistry, Martinsried, Germany. ⁵Clinical Proteomics Group, Proteomics Program, Novo Nordisk Foundation Center for Protein Research, University of Copenhagen, Copenhagen, Denmark.

*Present address: Laboratory of Systems Neuroscience, Department of Health Science and Technology, Institute for Neuroscience, Swiss Federal Institute of Technology, Zurich, Switzerland.

†Corresponding author. Email: steven.brown@pharma.uzh.ch (S.A.B.); charo.robles@med.uni-muenchen.de (M.S.R.); tyagarajan@pharma.uzh.ch (S.T.)

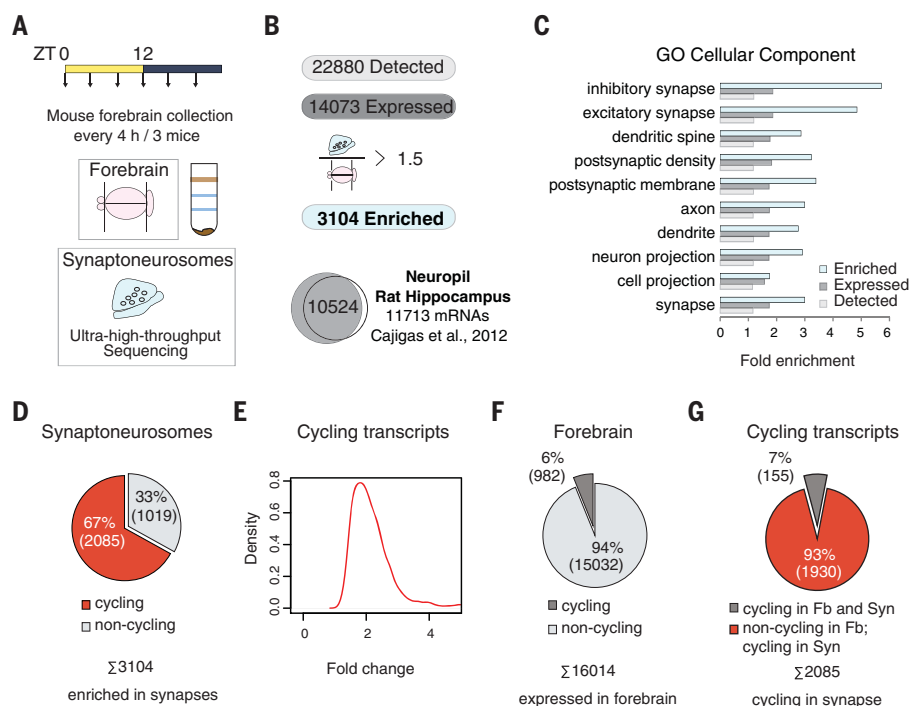


Fig. 1. Daily rhythms within the synaptic transcriptome. (A) Workflow: Forebrains from mice were collected in biological triplicates at six times throughout the day. Synaptoneurosomes were prepared, RNA isolated and sequenced, and data analysis was performed using Perseus software. (B) Number of transcripts from successive steps of the synaptoneurosome workflow. Synaptic transcripts were those 1.5-fold enriched in synapses versus total forebrain. Bottom: Detected transcripts correspond closely to those identified in synaptic neuropil (23). (C) Top 10 GO cellular component annotations. BH-adjusted $p < 0.001$, FunRich analysis (<http://www.funrich.org>) in the synaptic transcriptome. Bars show the calculated fold enrichment versus the mouse genome for synaptic-enriched (blue), detected (light gray), and expressed (dark gray) GO-associated transcripts. (D) Pie chart depicting the fraction of cycling mRNAs (Perseus time-series periodic analysis, period 24 hours, $q < 0.05$) among enriched synaptic mRNAs. (E) Density distribution of circadian amplitudes (peak/trough) of cycling synaptic transcripts from (D). (F) Fraction of cycling mRNAs (Perseus time-series periodic analysis, period 24 hours, $q < 0.05$) in the whole forebrain. (G) Fraction of circadian synaptic-enriched RNAs cycling only in synaptoneurosomes. Note that, of the synaptic cycling mRNAs ($n = 2085$), only 7% ($n = 155$) cycle in the forebrain and the synapse (gray) and 93% ($n = 1930$) cycle exclusively in synapses.

in the synaptic transcriptome are entirely or nearly entirely driven by posttranscriptional processes.

In principle, oscillations in the abundance of synaptic transcripts could arise either from transport of mRNAs or from local control of their stability. Both to verify our transcriptional results and to distinguish between these possibilities, we employed single-mRNA fluorescence in situ hybridization (FISH) using the RNAscope strategy (see materials and methods). As test cases, we selected the vesicular glutamate transporter *Slc17a7* (vesicular glutamate transporter 1) and *Lingo1* (leucine rich repeat and Ig domain containing 1), with previously reported synaptic function, high levels of expression in hippocampal cornu ammonis area 1 (CA1) and cortex (27, 28), and high amplitude in our circadian analysis. As a control, the circadian clock mRNA *Cry1* was also imaged.

Using custom automated imaging workflows (see materials and methods), we visualized and quantified axodendritic and somatic mRNAs separately in hundreds of images. Similar to previous reports (29), *Cry1* mRNA in the hippocampus increased toward the end of the dark period, and the same trend was observed in the cortex (fig. S3, A and B). For synaptic cycling mRNAs, robust oscillations were observed in the axodendritic compartment (Fig. 2A and fig. S4), with maximum levels at Zeitgeber time (ZT) 4 and trough levels at ZT12 in both CA1 and cortex (Fig. 2B). We further quantified CA1 dendritic structures according to distance from the pyramidal cell layer. Equal daily rhythms of mRNA abundance were observed at all distances (Fig. 2C). Even though synaptic density dramatically increases with distance (30), we found identical circadian amplitude at all distances, suggesting that the synaptic oscillations

of transcript abundance are likely generated through transport along the dendritic arbor. However, other explanations such as regulated RNA stability certainly also remain possible.

Synaptic oscillations anticipate dawn and dusk and depend on a functional clock

Cycling synaptic transcripts clustered entirely into two temporal categories, with maxima anticipating dawn or dusk (lights-on and lights-off in our laboratory scenario; Fig. 3A). Because these peaks of transcript accumulation anticipated light-dark transitions, we hypothesized that they were driven by a circadian clock. To verify this presumption, we did synaptoneurosome transcriptome analysis at two time points (ZT0 and ZT12) from mice kept in normal LD conditions, mice kept in constant darkness, or *Bmal1*-knockout mice (*Bmal1*^{-/-}), which lack an essential clock gene and therefore lack a functional circadian oscillator (31). Transcripts showing significant differences in abundance [exact binomial test, Benjamini–Hochberg (BH)-corrected $p < 0.05$] between the two times in wild-type mice under LD conditions (Fig. 3B; significant values are shown in red) also showed differences when kept in darkness (Fig. 3C; significant values are shown in dark gray). Conversely, no significant changes were observed for those transcripts in *Bmal1*^{-/-} mice (Fig. 3C; significant values are shown in green).

The two waves of transcript abundance that we detected were not only sharply segregated by phase but also entirely ontologically distinct (Fig. 4, A and B; fig. S5; and table S4). Moreover, the light and dark circadian synaptic clusters were further enriched in specific biological processes compared with the whole synaptic transcriptome (fig. S5 and table S4), emphasizing an important temporal and local regulation. mRNAs anticipating dusk participate in cellular pathways related to synapse organization, synaptic transmission, and higher functions relying on them directly, such as memory, learning, and behavioral outputs (Fig. 4A). Those anticipating dawn are required for metabolism, with a high representation of lipid catabolism, translation, and cell proliferation or development (Fig. 4B).

Coordination of daily mRNA oscillations by circadian clocks and sleep cycles

One major behavioral output of the circadian oscillator in brain is the sleep-wake cycle. At the level of the whole forebrain, it has been demonstrated previously that the vast majority of “circadian” transcription is actually sleep-wake driven (3). To arrive at this conclusion, the authors systematically deprived mice of sleep for the 6 hours preceding sacrifice at four different times of day during the circadian cycle, thereby keeping sleep pressure high across all samples (3, 32). By so doing, they observed that circadian oscillations of most brain transcripts

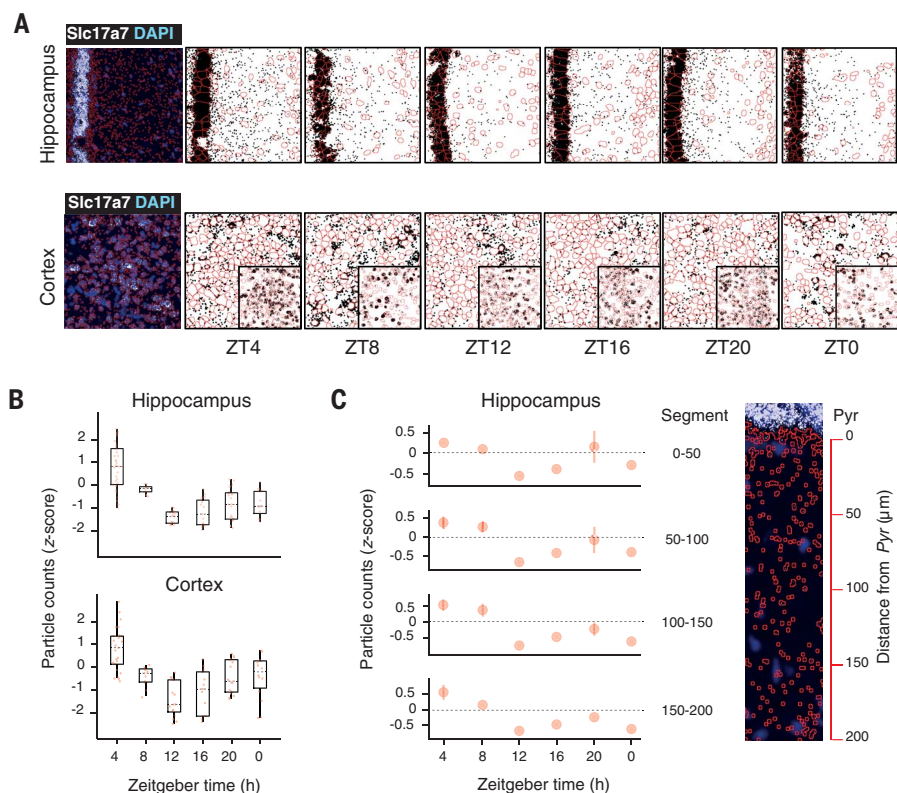


Fig. 2. mRNA FISH visualization of *Slc17a7* (*VGlut1*) diurnal abundance in hippocampus and cortex.

(A) Confirmation of rhythmicity by single-molecule fluorescence in situ hybridization (FISH) of *Slc17a7* (*VGlut1*) in the stratum radiatum of the CA1 of the hippocampus (top panels) and in the axodendritic compartment of cortex (lower panels). For better visualization, red line traces nuclei and single mRNA dots are increased to 0.5 μm . Insets: mRNA in the somatic areas of the cortex. (B) Quantification of mRNA abundances in (A) ($n = 13$ to 18 from three biological replicates). (C) Quantification of mRNA in stratum radiatum normalized and plotted as a function of distance from the pyramidal (Pyr) segments. (mean $\pm$ SD). Sample image is shown at right.

disappeared. To test whether synaptic mRNA oscillations are controlled by sleep pressure, we first derived an analogous protocol in which sleep pressure would be kept elevated across the circadian day at levels roughly equal to its normal 24-hour maximum under undisturbed conditions. Typically, sleep pressure is indicated by the amplitude of subsequent electroencephalogram (EEG) oscillations during sleep: The greater the sleep pressure, the greater the amplitude of “delta” oscillations (0.5 to 4 Hz) (32). As can be seen in Fig. 5, A and B, across six equally spaced time points during the day, 4 hours of prior sleep deprivation by gentle handling leaves at each time point a level of delta power approximating that maximally observed spontaneously in the day before the manipulation (top lines), without disrupting the circadian phase of sleep-wake behavior the following day (bottom lines). Although some fluctuation in delta power across time points is still observed, we estimate it to be less than one-fifth of that observed under the same conditions across the normal circadian day. At some times of day (e.g., the start of the day at ZT0 to

ZT4, when mice are sleeping; fig. S6, A and B), this sleep deprivation results in a significant decrease in sleep latency (the time to fall asleep, fig. S6C) and increase in delta power (Fig. 5, A and B) relative to control conditions. At other times (e.g., the start of the night at ZT12 to ZT16, when mice would normally be awake anyway; fig. S6, A and B), the same sleep deprivation results in almost no changes relative to control conditions (Fig. 5, A and B, and fig. S6, C and D). In all cases, subsequent sleep and activity are completely normal, with no shift in the timing of activity (Fig. 5, A and B).

We next performed this protocol preceding each time point of our synaptoneurosome transcriptomics (Fig. 5, A and B). Consistent with observations of the whole-brain transcriptome by other investigators (3), the rhythmicity of a large proportion of mRNAs in synaptoneurosomes was significantly altered by sleep deprivation. In general, cycling features in baseline (BL) conditions showed reduced statistical significance (higher q values) under sleep deprivation (SD) (Fig. 5C and table S5). However, circadian oscillations of one-fourth (561) of

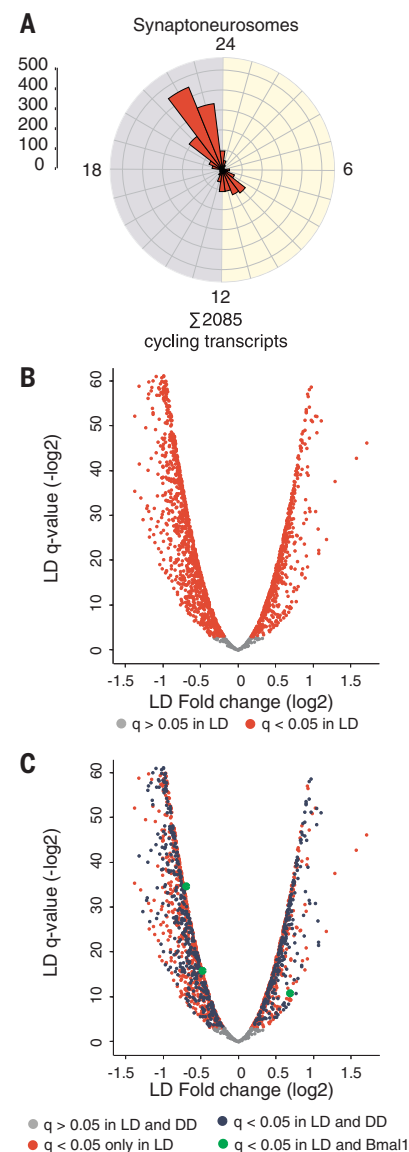


Fig. 3. Synaptic RNA abundance anticipates dawn and dusk and depends on a functional circadian clock.

(A) Phase distribution of the rhythmic synaptic transcriptome in LD. Angular axis, Zeitgeber time; magnitude, number of transcripts peaking at indicated time. (B) Volcano plot representing the fold change and q value between ZT0 and ZT12 for the total synaptic rhythmic transcriptome from mice kept under LD conditions (12:12). Red dots, mRNAs with abundance significantly different between times (t test, $q < 0.05$, $n = 3$); gray dots, mRNAs not significantly different. (C) Volcano plot as in (B), where mRNAs are labeled as follows: red, significant differences between ZT0 and ZT12 only (i.e., in LD conditions only); blue, significant differences between CT0 and CT12 as well (i.e., after 48 hours of dark:dark conditions); green, significant differences between the same time points in *Bmal1*^{-/-} clock-deficient mice after 48 hours of dark:dark conditions. For visualization purposes, the three significant dots were enlarged ($n = 3$ mice).

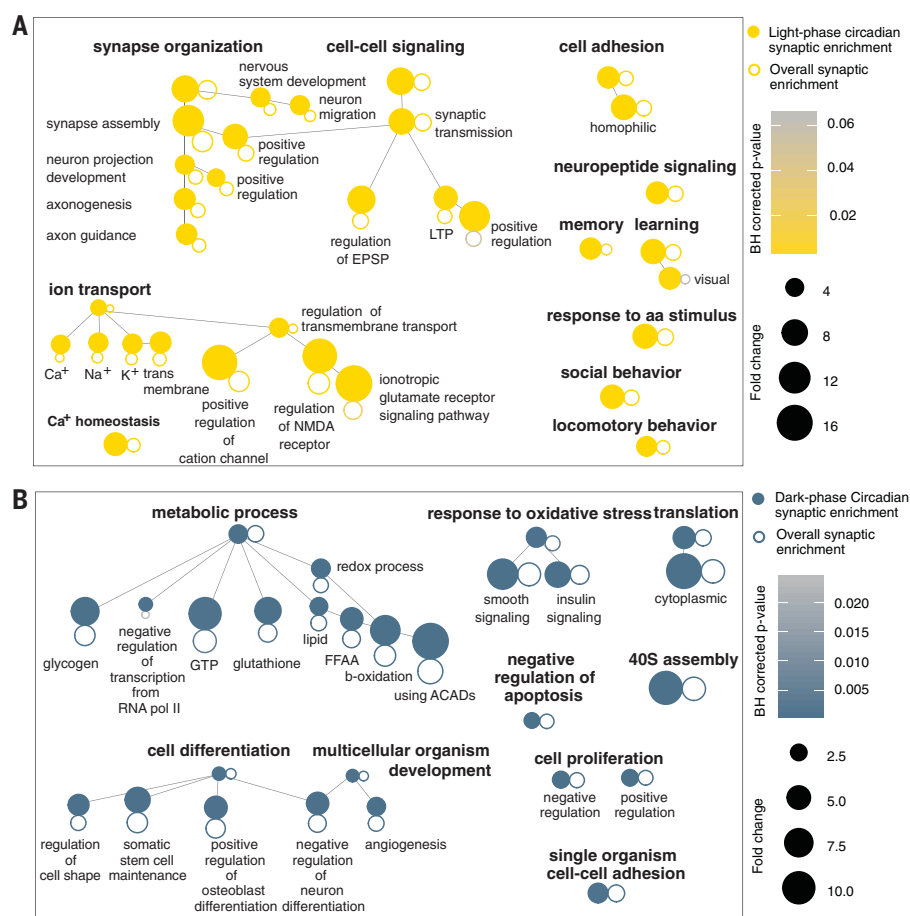


Fig. 4. Predawn and predusk mRNAs relate to specific and distinct functions. (A) GO analysis of biological processes enriched in the light (predusk) mRNA cluster (725 genes), grouped according to ontological hierarchy. Circle size is proportional to the fold enrichment and color is proportional to significance. Higher-level annotations are in bold. BH-corrected $p < 0.001$, FunRich analysis (<http://www.funrich.org>). Open circles, enrichment for the same terms in the overall synaptic transcriptome. (Note the decrease in fold enrichment and significance for most annotations.) (B) Same as in (A), but for the dark peaking (predawn) mRNA cluster ($n = 1217$ genes).

synaptic mRNAs were preserved (period 24 hours, $q < 0.05$) and virtually unchanged in amplitude compared with BL conditions (Fig. 5, D to F, and fig. S7A), and of the remaining 1524 transcripts, 1271 still showed profiles with considerable time-of-day-dependent variation (Fig. 5G and fig. S7B). Ontologically, the analysis of rhythmic mRNAs presents a picture that is mostly unchanged (table S6). mRNAs that encode proteins involved in synaptic transmission are enriched in the peak before the transitions to the wake phase (Fig. 6A). By contrast, mRNAs peaking before dawn, preceding the sleep phase, are involved in intracellular signaling, cell morphology, cell metabolism, and translation (Fig. 6B).

Daily variations in the synaptic proteome are dominated by sleep-wake state

Previous studies suggested that mRNAs in the synapse are translated there, and half of these

locally translated transcripts were also present in our cycling dataset (24) (fig. S8). To gain further insights into the functional implication of daily oscillations of synaptic mRNAs, we performed MS-based, label-free quantitative proteomics to characterize temporal patterns of the total forebrain and synaptic proteome. We measured, in a single-shot manner, protein samples prepared from isolated synaptoneurosome and total forebrain of mice (four biological replicates) collected every 4 hours across 24 hours (see materials and methods). Our in-depth analysis allowed us to quantify across all samples 4477 proteins in total forebrain and 4063 in synapses, with an overlap of 3710 proteins (fig. S9A). Circadian analysis revealed that in synapses, 11.7% of proteins (476; Fig. 7A and table S7A) and in total forebrain, 17.2% of proteins (770; fig. S9B) were rhythmic (period 24 hours, $q < 0.1$; table S7B). [Note that proteomic analyses showed lower circadian range and

relative signal for less abundant components than transcriptomics; the q value was set at 0.1 at the maximum of the circadian q -value distribution to ensure comparable coverage and comparability with transcriptomics (fig. S9, C and D). Analyses identical to those in Fig. 7 are also presented in fig. S10, with a q value set at 0.05, arriving at the same conclusions with smaller numbers of proteins.]

Although both the cycling synaptic and fore-brain proteomes showed biphasic distributions, these were markedly different (fig. S11, A and B, and table S7C), with peak phases differing across compartments by 6 hours (fig. S11, C and D). Moreover, from the common proteins in both datasets (fig. S9A), only 92 were cycling in both forebrain and synapse, and these also had substantially different phases of maximal expression (Fig. 7, B and C, and fig. S11, E and F), suggesting, as for the transcriptome, totally different mechanisms for daily protein rhythm generation in the two compartments.

By contrast, the phase distribution of synaptic cycling proteins mirrored that observed for oscillating synaptic transcripts, with two clusters preceding dusk and dawn (fig. S11, B and D). We detected synaptic mRNAs for 1128 synaptic proteins (fig. S12A) and, of those with daily oscillations (fig. S12B), 77.7% also had a cycling transcript predominantly with a leading or shared phase (Fig. 7, D and E). As revealed for transcripts, proteins peaking before dawn were enriched in categories related to metabolism, and more specifically to lipid metabolism and mitochondria, whereas proteins involved in cellular signaling preceded dusk (Fig. 7, F and G, and table S8). Thus, under normal conditions of light and dark, the temporal profiles of the synaptic proteome largely resembled those of the transcriptome.

Analogously to the transcriptome, we next examined the daily cycles in the proteome under conditions of high sleep pressure. Here, a markedly different picture emerged. In the time course from the serial sleep deprivation, almost all (98% with a cutoff of $q < 0.1$ and 99.9% with a cutoff of $q < 0.05$) of the oscillating proteins in BL lost their rhythms (period 24 hours; Fig. 8, A to C, and table S9). Our data indicate that daily changes in protein levels at the synapse are completely determined by vigilance state rather than by circadian clocks.

Discussion

In various mammalian tissues, between 3 and 16% of total transcripts have been described as circadian until recently (33), and our own figure of 6% of oscillatory mRNAs in the whole mouse forebrain is consistent with these values. However, synaptic functionality has been shown to have a strong circadian component. In the hippocampus, long-term potentiation efficacy undergoes circadian variation (34), and in the SCN, dynamic expression and function of ion

Fig. 5. Circadian and sleep-wake regulation of synaptic mRNA abundance.

(A and B) Mean ($\pm$ SEM) time course of EEG relative (to mean BL delta power; 0.5 to 4 Hz at ZT8 to ZT12) frontal delta power during NREM sleep (triangles; percentage of the last 4 hours of the BL light periods) and time spent in NREM sleep (circles; minutes/recording hours; left y-axis) during 24 hours of BL (gray symbols and lines), 24 hours of recovery, and during and after 4-hour SD finishing at ZT4 (red, sample name BL4/SD4), ZT8 (orange, sample name BL8/SD8), ZT12 (yellow, sample name BL12/SD12), ZT16 (green, sample name BL16/SD16), ZT20 (blue, sample name BL20/SD20), or ZT0 (purple, sample name BL0/SD0). Gray areas delineate the dark periods. The data was divided into two panels for better visualization but the baseline is the same in both.

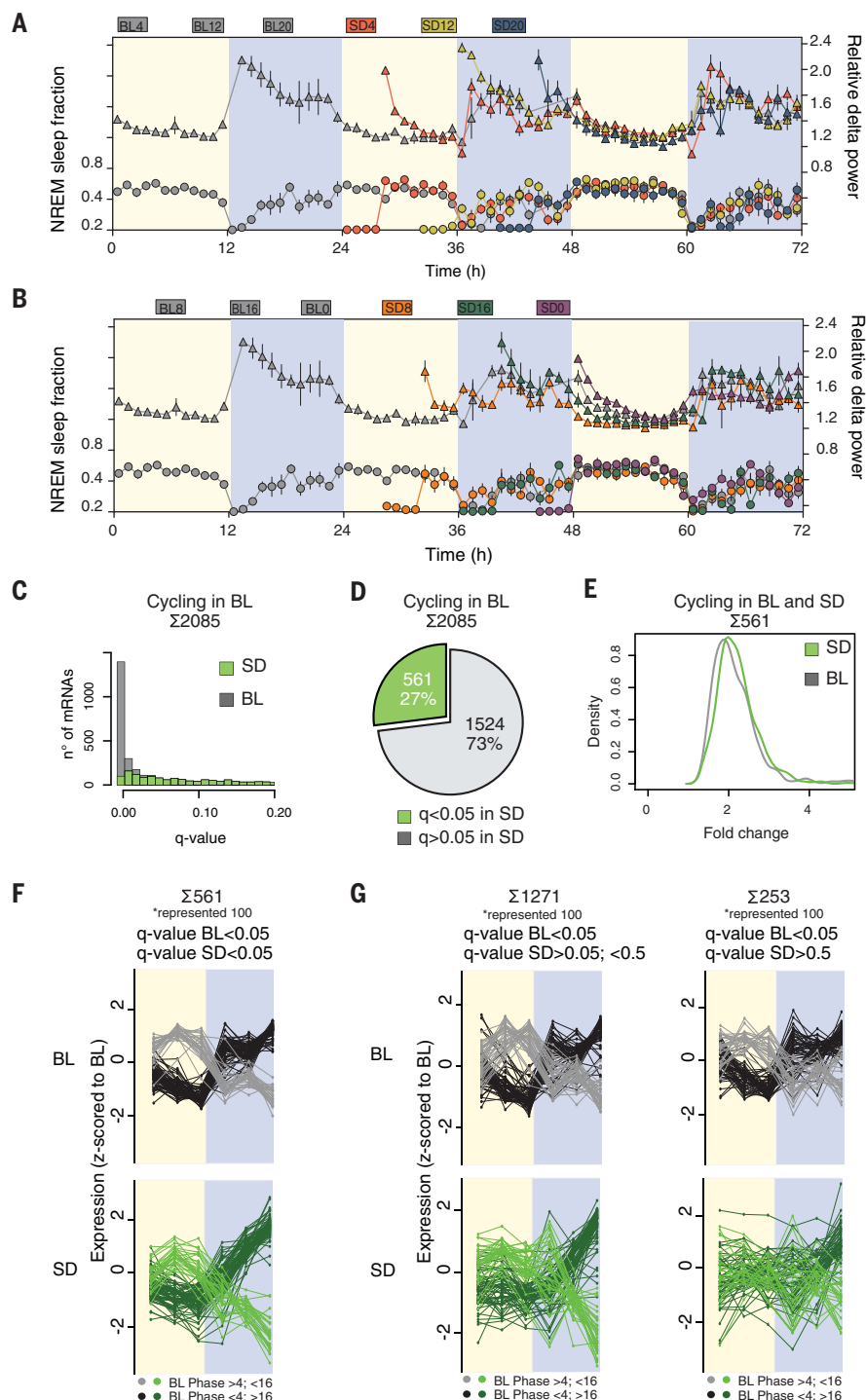
(C) Distribution of q values (Perseus time-series periodic analysis, period 24 hours) of the 2085 mRNAs cycling in BL (in red are q values at BL, in green are q values after SD). q values > 0.5 were omitted.

(D) Pie chart depicting the fraction of mRNAs that remain rhythmic (Perseus time-series periodic analysis, period 24 hours, $q < 0.05$) in synaptoneurosome of SD mice.

(E) Density distribution of circadian amplitudes (peak/trough) of cycling synaptic transcripts that remain rhythmic after SD. In gray is the amplitude in BL and in green the amplitude in SD.

(F) Expression profiles of transcripts cycling in BL and SD (q -value BL < 0.05 and q -value SD < 0.05 ; 561 mRNAs). One hundred randomly selected mRNAs are shown.

(G) As in (F), but for those transcripts cycling in BL ($q < 0.05$) but nonsignificant in SD; $n = 1271$ transcripts with lower q values in SD ($0.05 < q < 0.5$; left) and $n = 253$ transcripts with higher q values in SD ($q > 0.5$; right) are represented separately. One hundred randomly selected mRNAs are shown.



channels or neurotransmitter receptors ensure distinct effects of light depending on time of day (4, 35–37). Helping to achieve this circadian functionality, we find that synaptic transcript accumulation can occur with much higher rhythmicity: 67% of enriched synaptoneurosome transcripts show time-of-day variation, encompassing all aspects of synaptic function. (It should be noted that other transcripts present but not specifically enriched at synapses do not

display this overwhelming degree of rhythmicity, implying differences in stability, circadian active transport, or both.)

Posttranscriptional mechanisms as a primary circadian motor

Statistically, our results further show that this rhythmicity is generated at a posttranscriptional level, because overlap between the set of synaptic cycling transcripts and that in the whole

forebrain is at levels expected by chance alone. This observation is consistent with several recent studies showing circadian variation at steps subsequent to transcription initiation (5, 11, 38, 39). In the brain, we observe rhythms of transcript abundance microscopically at equal amplitude throughout the axodendritic arbor in the stratum radiatum of the hippocampus despite marked variation in synaptic density. Thus, spine-poor proximal regions (30) show the same amplitude

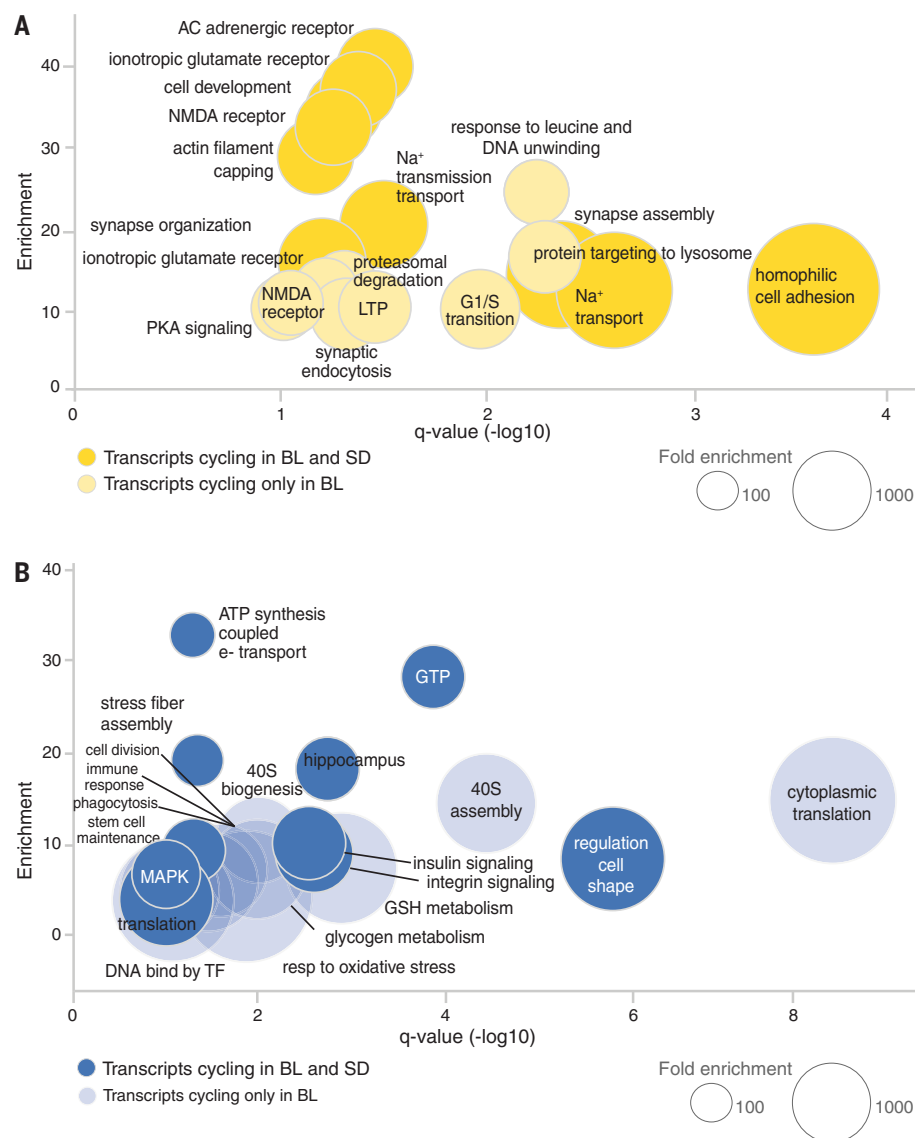


Fig. 6. Synaptic accumulation of RNA preceding dawn and dusk is independent of sleep pressure.

(A) GO analysis of biological processes enriched in the light (predusk) peak (according to phase in BL). In dark yellow are transcripts cycling in both BL and SD; in light yellow are those cycling significantly in BL but nonsignificantly in SD (light yellow). Size of the circle indicates fold enrichment in the light versus dark. (B) Same as in (A), but for the dark peak, with circle size indicating enrichment in dark versus light.

as spine-rich distal regions. For this reason, we favor the hypothesis that the generation of synaptic transcript daily oscillations happens owing to cyclical transport to synapses. Dynamics of cytoskeleton components have already been linked to the circadian clock (40) and represent a possible regulatory node for transport in the cytoplasm. However, other interesting posttranscriptional steps could be involved, including changes in the RNA degradation rate within the dendritic arbor or at synapses. Because messenger ribonucleoproteins remain docked at the nuclear basket for an uncertain time, constituting a rate-limiting step for mRNA dynamics (41, 42), export through the nuclear

pore is also an appealing regulatory step for circadian regulation of mRNA abundance outside the nucleus.

A similar lack of parallelism between synapse and whole forebrain can be observed when examining the proteome. Three-quarters of cycling synaptic proteins arise from cycling synaptic transcripts, but only 20% of proteins show oscillations in the whole brain. It is difficult to draw conclusions about the relative percentages of oscillatory transcripts and proteins because different platforms were used for data generation (deep sequencing versus mass spectrometry). However, our results are consistent with the reported stability of many synaptic

proteins (19), which would correspondingly decrease their oscillatory amplitude. There is also a small difference in overall phase angle between the two datasets. This phase discrepancy suggests that local translation of synaptic proteins could be a major rate-limiting step for daily changes in synaptic protein levels. Supporting this idea, a recent study in neuronal culture demonstrates that mRNA localization in the synapse is the primary mechanism contributing to the synaptic proteome compared with transport of proteins synthesized in the soma (43).

A major division of biological functions preparing for sleep and wake

In most mammalian organs, broad peaks of gene expression precede dusk and dawn (2). In our own study, nearly without exception, both circadian transcripts and the proteins derived from them showed peak levels sharply in anticipation of dusk and dawn. Transcripts in these two phases showed a complete division of biological function: cell-intrinsic and metabolic ontological terms preceded sleep, whereas terms associated with synaptic structure and function preceded wake. The division that we observed is consistent with a large literature suggesting both circadian- and sleep-dependent partition of cellular function. An inherent circadian clock is known to regulate learning and memory efficiencies diurnally (44–47). Mechanistically, it has been proposed that trafficking of glutamate receptors or modulation of spine densities could be involved (48–50). Along the same lines, synaptic homeostasis has been proposed as a major function of sleep-wake states (20), and others have proposed macromolecular synthesis and energy replenishment as potential functions for sleep (51, 52). Our synaptic GO data are consistent with both suggested functions of sleep-wake cycles, neatly partitioned in circadian time. In a companion paper (53), we further document the role of synaptic protein phosphorylation across the circadian cycle. Again, we found a purely bimodal distribution of these ontological states, though with opposite relative proportions: whereas the majority of cycling RNAs and proteins reach peak levels at the end of the wake period in anticipation of dawn, the majority of circadian phosphosites reach peak levels at the end of the sleep period in anticipation of dusk (53).

Transcript timing is dominated by clocks, protein timing by sleep-wake state

Because of the coupling of circadian rhythms and sleep-wake cycles, distinguishing the contribution of each to cellular biology remains challenging. By depriving mice of sleep before each time point, one mostly dampens the normal diurnal variation of sleep need: during the light phase, SD induces an increase in sleep pressure, whereas during the dark phase, no significant

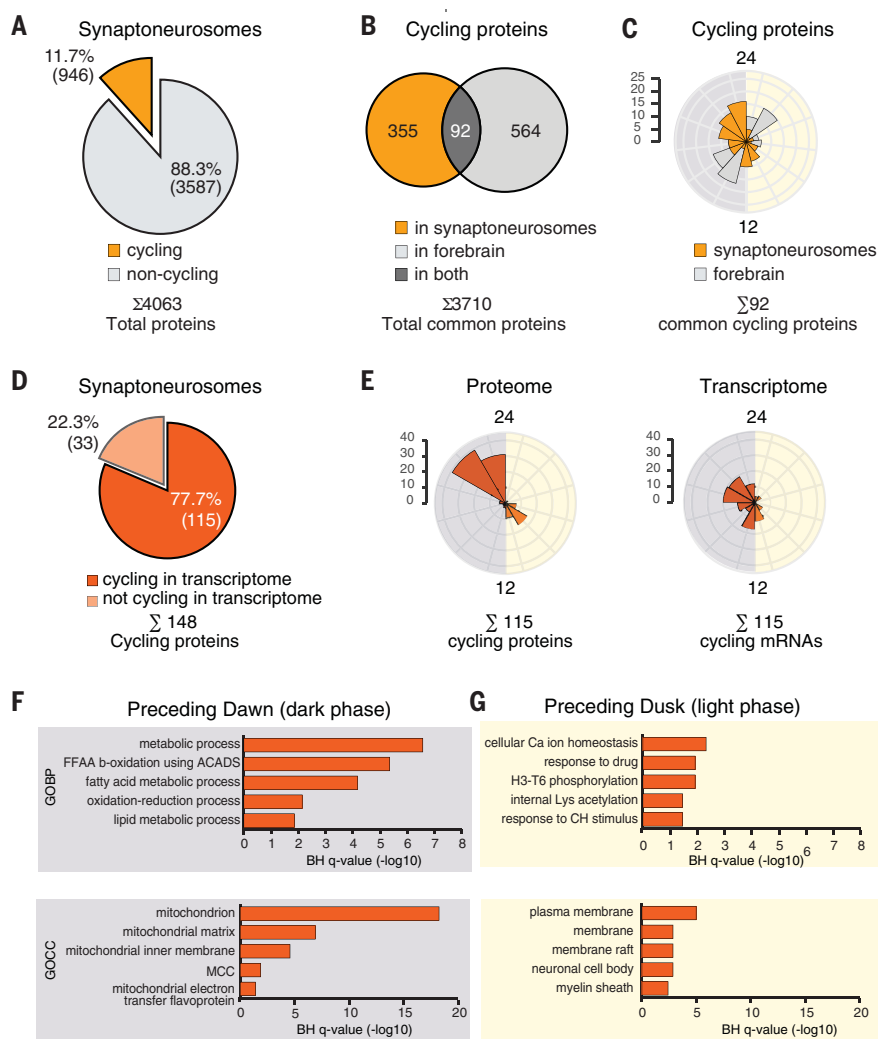


Fig. 7. Oscillations of the synaptic proteome resemble those of the synaptic transcriptome. (A) Pie chart showing the fraction of cycling proteins (Perseus time-series periodic analysis, period 24 hours, $q < 0.1$) in synaptoneurosomes among the total 4063 quantified. (B) Overlap of the rhythmic proteome in synapses and in total forebrain from those quantified in both compartments ($n = 3710$ proteins). (C) Phase distribution in synapses (orange) and forebrain (gray) of proteins cycling in both compartments. (D) Proportion of synaptic cycling proteins with synaptic cycling mRNAs. (E) Rose plots representing the frequency distribution of phases for cycling proteins and corresponding transcripts at synapses. (F and G) Enriched GO Biological Processes (GOBP) and GO Cellular Components (GOCC) in the cycling synaptic proteome, with cycling transcripts separated according to protein phase: dark, anticipating dawn (F) and light, and anticipating dusk (G). BH-corrected $p < 0.01$, FunRich analysis (<http://www.funrich.org>).

difference occurs because animals are already awake (Fig. 5A and fig. S6) (32). Under these conditions, circadian transcriptional oscillations of the mouse forebrain are strongly dampened (3). At the synapse, the posttranscriptional mechanisms dominating diurnal transcript accumulation are less affected: whereas circadian rhythmicity is globally decreased, one-fourth of circadian transcripts remain completely unchanged, and most others retain some evidence of time-dependent variation.

These time-of-day-dependent oscillations in synaptic transcript abundance persist in constant darkness, ruling out light-driven effects, but are lost in circadian clock-deficient *Bmal1*^{-/-}

mice in entrained LD conditions. It is thus tempting to attribute cyclical abundance purely to circadian clock control. However, *Bmal1*^{-/-} mice (54), as well as all other clock-depleted strains tested (55), show markedly dampened daily variation in sleep pressure across the day, even under normal LD conditions. Therefore, our experiments do not rule out clock:sleep interactions, even if they demonstrate total dependence of cyclic synaptic RNA accumulation upon clock function.

Ontological terms related to synaptic organization and assembly, cell adhesion, and actin cytoskeleton all retain high statistical significance and synaptic enrichment equally under

conditions of sleep deprivation. Thus, sleep-wake cycle-dependent changes in synaptic structure (20, 56, 57) appear to be preceded by a primarily circadian-driven accumulation of relevant RNAs.

At the protein level, almost no circadian influence remains under conditions of high sleep pressure. Although daily cycles of proteins in the synapse are detected in animals kept under an LD cycle, sleep deprivation completely blunts those changes.

Our findings are consistent with cellular literature suggesting activity-dependent translation at synapses: new experience triggers the association of mRNAs to ribosomes in the synapse, synaptic activation rapidly releases the translational repression of mRNAs localized in the synapse, and local protein translation is essential for several forms of plasticity that involve active behavior (58–61). Similarly, evidence of synaptic downscaling during sleep has been observed ultrastructurally in mouse cortex (56) and likely occurs because of metabotropic glutamate receptor 5 (mGluR5)-dependent signaling during waking (19). Thus, under the serial SD protocol that we used, as oscillations in protein levels are dampened, protein levels are mostly driven toward peak rather than nadir levels.

Many possibilities exist for the upstream signaling that drives the sleep- and circadian-dependent accumulation of RNA and proteins that we observed. Whereas the mechanism connecting cellular components of the circadian oscillator to downstream pathways is well established (62, 63), neither the workings of the sleep homeostat itself nor its connection to downstream sleep-dependent cellular events has been deciphered. In addition to the direct synaptic activity-dependent hypotheses that we favor (20), indirect mechanisms such as changes in brain temperature and cortisol levels are also possible. Changes in transcript abundance associated with small alterations in body temperature are believed to be mediated either by an initial posttranscriptional effect by the cold-inducible RNA-binding protein CIRBP (64) or by low-level activation of Heat Shock Factor 1 (65). We have found no overlap between mRNAs regulated by these factors and our cycling dataset, making it unlikely that temperature changes are responsible for the effects that we observed. Similarly, although cortisol elevations during wake and especially during sleep deprivation are well documented, adrenalectomy experiments establish that cortisol is not responsible for most of the transcriptional effects of SD (66). Therefore, we also disfavor this indirect cue as a primary signal.

Overall, our results are consistent with anticipatory circadian delivery of synaptic mRNAs before dawn and dusk, followed by “need-dependent” local translation linked to sleep and wake states. These spatiotemporal dynamics across the synaptic landscape likely play a

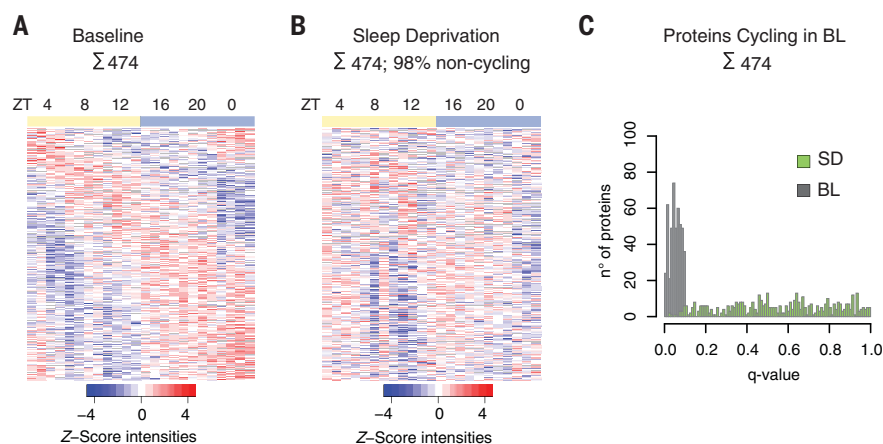


Fig. 8. The synaptic proteome shows oscillations almost entirely dependent upon sleep-wake state. (A and B) Heatmap of label-free intensities of cycling proteins in BL (A) and the corresponding values in SD (B) in all biological replicates for each sampled time point (columns). Proteins (in rows) are ordered by the estimated phase in BL, and all intensities in both conditions were z-scored to BL intensity values. (Note: of the 474 proteins represented, 98% did not cycle in SD.) (C) Distribution of the q values (Perseus time-series periodic analysis, period 24 hours) of the cycling synaptic proteome in BL (gray) and the corresponding q values in the SD dataset (green).

critical role in diurnally regulating all aspects of forebrain function.

Materials and Methods

Animals and tissue collection

All experiments were performed after approval by the animal welfare officer of the University of Zürich and veterinary authorities of the Canton of Zürich. Ten-week-old male C57BL/6 mice were housed with free access to food and water and entrained to a 12 hour:12 hour LD schedule for 14 days. Mice were sacrificed at 4-hour intervals over 1 day (ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20; $n = 3$ mice for transcriptome and $n = 4$ mice for proteome). At the time points overlapping with light transitions (ZT0 and ZT12), euthanasia was performed right before the light change. For the around-the-clock sleep deprivation, mice were allowed to acclimatize to a 12-hour light/12-hour dark cycle for 14 days. Six groups of mice were sleep deprived for 4 hours by gentle handling [cage exchange and introduction of new objects as described previously (67)] at different times of day [SD4 (sleep deprivation from ZT0 to ZT4), SD8, SD12, SD16, SD20, and SD0; $n = 3$ mice for transcriptome and $n = 4$ mice for proteome]. To collect synaptoneurosomes from mice kept in constant darkness and from *Bmal1*^{-/-} mice, animals were kept in LD for 2 weeks and then transferred to constant darkness (DD) 48 hours before euthanasia at circadian times (CT) CT0 and CT12, respectively ($n = 3$).

EEG recording and sleep data analysis

Adult C57BL/6 mice were used for surgery (8 to 10 weeks old at surgery). Mice were implanted epidurally under isoflurane anesthesia for EEG recording. Right before and 24 hours after

surgery, mice were treated with an analgesic (Temgesic, 0.1 mg/kg, intraperitoneal). Gold-plated miniature screws (0.9-mm diameter) were used as EEG electrodes and positioned on the left hemisphere above the frontal cortex (1.5 mm anterior to bregma, 1.5 mm lateral to the midline) and the parietal cortex (2 mm posterior to bregma, 2 mm lateral to the midline). The reference electrode was placed above the cerebellum (2 mm posterior to lambda, 0 mm lateral to the midline). Screws were connected to copper wires and fixed to the skull with dental cement (Paladur two-component system). Electromyography (EMG) was recorded using two gold wires (0.2-mm diameter) inserted bilaterally in the neck muscle. After 1 week of recovery, EEG and EMG were recorded continuously for 7 days. Two cohorts of six and eight mice, respectively, underwent 1 day of BL recording and 3 days of SD recording, with 48 hours of recovery in between. Cohort 1 underwent SD at ZT4 to ZT8, ZT12 to ZT16, and ZT16 to ZT20. Cohort 2 underwent SD at ZT0 to ZT4, ZT8 to ZT12, and ZT20 to ZT24. SD was performed by gentle handling as described previously (67). Both EEG and EMG signals were amplified (factor 2000), analog filtered (high-pass filter: -3 dB at 0.016 Hz; low-pass filter: -3 dB at 40 Hz, <-35 dB at 128 Hz), sampled with 512 Hz, digitally filtered (EEG, low-pass FIR filter: 25 Hz; EMG, band-pass FIR filter: 20 to 50 Hz), and stored with a 128-Hz resolution. EEG power spectra were computed for 4-s epochs by a fast Fourier transform routine within the frequency range of 0.5 to 25 Hz. Between 0.5 and 5 Hz, 0.5-Hz bins were used, and between 5 and 25 Hz, 1-Hz bins were used. The corresponding slow-wave-activity (SWA) was calculated using the raw parietal and fron-

tal EEG, as well as the raw and integrated EMG, to visually score three vigilance states: non-rapid eye movement (NREM) sleep, rapid eye movement sleep (REM), and wake, for 4-s epochs. Epochs containing artifacts were identified and excluded from the spectral analysis. Data analysis was carried out using MATLAB version R2015a (The MathWorks, Inc., Natick, MA, USA). Relative frontal SWA was calculated relative to the mean SWA at ZT8 to ZT12 during the BL day. Sleep loss was calculated by comparing NREM sleep amount in each 4-h SD slot with the sleep amount in the same time of day of the corresponding BL day [$p < 0.05$, one-way analysis of variance (ANOVA)]. Sleep latency was analyzed by measuring the time each mouse stayed awake after the end of each 4-h SD until it slept for >1 min ($p < 0.05$, one-way ANOVA).

Purification of synaptoneurosomes

Synaptoneurosomes from mouse forebrain were prepared as described previously (22). In brief, brain was isolated and rapidly cooled to 4°C, washed in ice-cooled sucrose buffer (320 mM sucrose, 5 mM HEPES, pH 7.4), followed by homogenization with a Teflon-glass tissue grinder using a motor-driven pestle keeping samples cooled. Homogenate was centrifuged at 1000g for 10 min. Two milliliters of the supernatant was loaded over discontinuous Percoll gradients (3, 10, and 23% Percoll in sucrose buffer) and centrifuged at 31,000g for 5 min. The fractions at the interfaces between 3 and 10% and 10 and 23% were collected and further centrifuged at 20,000g to pellet synaptoneurosomes. All centrifugation steps were performed at 4°C. All solutions were supplemented with complete protease inhibitor cocktail (Roche), 0.05 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride, and 20 U/10 μ l RNaseOUT (Invitrogen).

RNA sample preparation

Two hundred microliters of homogenate was used for total RNA extraction. Briefly, tissue lysate in QIAzol Lysis Reagent (Qiagen) was vortexed for few seconds. Then, 0.2 ml of chloroform was added to lysate and mixed by vigorous shaking for 15 s. The mixture was incubated at room temperature for 10 min and centrifuged at 18,000g for 20 min at 4°C to separate phases. The upper aqueous phase containing RNA was carefully aspirated to a fresh, nuclease-free tube. The RNA was then precipitated by adding one volume of isopropanol and centrifuged at 18,000g for 20 min at 4°C. The RNA pellet was washed with 70% ethanol, air-dried, and dissolved in nuclease-free water.

Frozen synaptoneurosomes pellets (~500 μ l) were processed with the High Pure RNA Isolation Kit (Roche). Samples were mixed with 600 μ l of lysis binding buffer and further steps were performed according to the manufacturer's instructions. The dissolved total RNA was

quantified by NanoDrop (NanoDrop Technologies) and a Qubit (1.0) Fluorometer (Life Technologies, Pleasanton, CA, USA). The quality was assessed with a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

Library preparation, sequencing, and data processing

Poly(A) RNA sequencing was performed using 500 µg of total RNA. Strand-specific cDNA libraries were prepared using Illumina's TruSeq Stranded Sample Prep Kit (125-bp single-read mode) following the manufacturer's directions, then sequenced on a HiSeq 4000 (Illumina Inc., San Diego, CA, USA). The raw reads were first cleaned by removing adapter sequences, trimming low-quality ends, and filtering reads with low quality (phred quality <20) using Trimmomatic (versions 0.33 and 0.36) (68). Specific quality control measures were evaluated and the following samples were excluded: replicate number 3 in ZT16 BL because of degradation during the library preparation; one sample from ZT8 BL because of reduced read counts (<10 million); and two samples from ZT4 SD, three from ZT16 SD, and three from ZT20 SD because of signs of contamination with external material. Sequence alignment of the resulting high-quality reads to the mouse reference genome (build GRCm38) and quantification of gene-level expression was performed using RSEM (versions 1.2.22 and 1.3.0) (69). For downstream analysis, the mRNA features were filtered according to normalized (log mean) feature counts, which represent aggregated raw counts of mapped reads at the gene level (RSEM). We determined a threshold for minimum gene expression on the basis of the assumption that a transcript with >10 counts in two of three replicates is expressed (linear signal threshold of 10).

Bioinformatic and statistical analyses of transcriptomics data

To search for transcripts that are enriched in neuronal synapses, we did a differential gene expression analysis using a pairwise comparison between synaptoneuroosomes and whole brain at two time points, ZT0 and ZT12. The threshold to consider a gene enriched in the synapse was a fold change (of the synaptic samples versus the whole forebrain samples) >1.5 in one of the two time points.

Cycling analysis was performed using the computational platform Perseus (25). We fit the normalized mRNA counts to a cosine with a fixed period of 24 hours and with amplitude and phase as free parameters (9). Profiles were ranked by their variance ratio. This was the part of the variance explained by the fit divided by the contribution to the variance that was not accounted for by the fit. On the basis of this ranking, we determined a permutation-based false discovery rate (FDR) by repeating the

same procedure 1000 times on the same profiles but with randomized time labels. We used a statistical cutoff of $q < 0.05$ to define the cycling transcriptome. Hierarchical clustering was performed in a phase-preserving manner by restricting the order of elements to that determined by the output of the cosine model-based fitting. Amplitudes were calculated as the $\log_2$ of the fold change of counts. To evaluate the effect of constant darkness and the lack of a functional clock on the generation of the peaks of synaptic transcript accumulation, we compared the following conditions: ZT0 versus CT0, ZT0 versus CT0 *Bmal1*^{-/-}, ZT12 versus CT12, and ZT12 versus CT12 *Bmal1*^{-/-}, by applying a count-based negative binomial model implemented in the software package EdgeR (R version: 3.4.2, EdgeR version: 3.20.1) (70). The differential expression was assessed using an exact test adapted for overdispersed data. Genes showing altered expression with an adjusted (BH method) $p < 0.05$ were considered differentially expressed.

Protein sample preparation

For protein extraction, 4% SDS was added to each synaptoneuroosomal sample or to the homogenate, followed by 5 min of incubation at 95°C. Samples were flash frozen and stored at -80°C until used. Samples were lysed (0.1 M Tris-HCl, pH 7.6, and 4% SDS), sonicated in a Bioruptor (Diagenode) at 4°C for 15 min or until homogeneous suspension was formed, and boiled at 95°C for 5 min. Protein lysates were treated first with 1 µl of DTT (1 M), followed by 10 µl 2-chloroacetamide (0.5 M). Each treatment was performed at room temperature (22°C) for 20 min. The lysates were precipitated with acetone and protein digested as described previously (9). In detail, pellets were resuspended in 500 µl of trifluoroethanol digestion buffer. For protein digestion 1:100 (protein:enzyme) trypsin and LysC were added and samples incubated overnight at 37°C with rapid agitation (1500 rpm). Digested peptides were concentrated in a SpeedVac for 15 min at 45°C, followed by acidification using 10 µl of 10% trifluoroacetic acid (TFA). Peptides were then desalted using StageTips with two layers of styrenedivinylbenzene-reversed phase sulfonated (SDB-RPS; 3M Empore), washed twice with wash buffer (0.2% TFA), and then washed once with isopropanol containing 1% TFA. Peptides were eluted by adding 60 µl of SDB-RPS elution buffer [80% acetonitrile, 1.25% NH₄OH (25% high-performance liquid chromatography grade)] and immediately concentrated in a SpeedVac for 30 min at 45°C. Concentrated peptides were then resuspended in a buffer containing 2% ACN and 0.1% TFA before chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS analysis and data processing

Samples were measured in a single-shot manner (71), loading ~1 µg of peptide mixture onto a

50-cm reversed-phase column (diameter 75 µm; packed in-house with 1.9 µm C18 ReproSil particles; Dr. Maisch GmbH). The temperature of the homemade column oven was maintained at 60°C. The column was mounted to the EASY-nLC 1200 system (Thermo Fisher Scientific). The peptides were eluted with a binary buffer system consisting of buffer A (0.1% formic acid) and buffer B (80% ACN and 0.1% formic acid). A gradient length of 140 min was chosen (5 to 65% buffer B for 130 min followed by 10 min of 80% buffer B) with a flow rate of 300 nL/min. Peptides were then electrosprayed into a Q Exactive HF mass spectrometer (MS) (Thermo Fisher Scientific), obtaining full scans (300 to 1650 m/z , $R = 60,000$ at 200 m/z) at a target of 3×10^6 ions. The 15 most abundant ions were selected and fragmented with higher-energy collisional dissociation (target 1×10^5 ions, maximum injection time 60 ms, isolation window 1.4 m/z , underfill ratio 1%), followed by detection in the Orbitrap ($R = 15,000$ at 200 m/z). Raw MS data files were processed using MaxQuant [version 1.5.5.6] to calculate label-free intensities with the Andromeda search engine with FDR < 0.01 at the protein and peptide levels. The default settings were used with the following modifications: (i) the variable modification methionine (M), acetylation (protein N terminus), and the fixed modification carbamidomethyl (C) were selected; (ii) only peptides with a minimal length of seven amino acids were considered; and (iii) the “match between run” option was enabled with a matching time window of 0.7 min. For protein and peptide identification, the UniProt database from mouse (September 2014) including 51,210 entries was used. Each raw file and replicate was treated as one independent experiment.

Bioinformatic and statistical analyses of proteomics data

Processed data were uploaded in Perseus software (25). First, reverse sequences and potential contaminants were removed. Then, the total dataset was $\log_2$ transformed and label free intensities were normalized in each sample by subtracting the media of all intensities in the same sample. Proteins without label free intensities in <10 samples were removed in both the synaptoneuroosomes (SD/BL) and total forebrain datasets. Replicates 1 and 4 of ZT4, replicate 4 of ZT8, and replicate 1 of ZT12 of the total brain homogenate were not considered because the protein quantification in these samples was limited.

Cycling analysis was done as for the transcriptomic data, in this case using label-free protein intensity values. Amplitudes were as well calculated as the $\log_2$ -fold change. For comparison of transcriptome and the proteome data, we matched datasets by gene name or Uniprot ID.

GO analysis

All ontological analyses were done with the enrichment analysis tool FunRich using the Hgcn gene symbols. For analysis of synaptic enriched mRNAs (3104, of which 2954 were available in the database), the GO option “GOTERM Cellular Component” was selected. A maximum p value of 0.001 was chosen to select only significant categories. For the evaluation of synaptic enrichment, we filtered for annotations containing the following terms: “synap*,” “project*,” “dendr*,” “axon,” or “spine.” The percentage of genes for each annotation and the fold enrichment in the three categories, identified, expressed, and enriched features, were used to validate the enrichment strategy. Only the top 10 annotations according to p value were included in the graphical representation; all returned annotations are shown in the supplementary tables. Enriched biological processes of the cycling transcriptome were identified separately for light (752, of which 725 were available in the “Biological Process” database feature in GO) or dark phases (1263, of which 1217 were available in the Biological Process database). Features were ascribed to each group using hierarchical clustering according to the temporal expression profile. Annotation with a BH-corrected $p < 0.001$ was included. Enrichment for biological processes in the synaptic enriched dataset was also performed for comparison of the common annotations. To compare the annotation enrichment between the light and dark clusters, we performed a fold enrichment analysis between both datasets and obtained a fold value (included in table S4). To analyze the gene datasets obtained after the SD experiment, we followed similar steps. Four groups of features were independently analyzed according to phase or cycling behavior in SD (light and $q < 0.05$, 149 features; light and $q > 0.05$, 603 features; dark and $q < 0.05$, 393 features; dark and $q > 0.05$, 863 features). For representation, the top 10 enriched annotations (ranked by the fold value between phases for cycling or not in SD and vice versa and with BH-corrected $p < 0.1$) were considered. For the common cycling features at the mRNA and transcript levels, the phase of protein was used to classify each into the light cluster (29 features) or dark cluster (27 features). For representation, we selected the top five enriched annotations (BH-corrected $p < 0.01$).

Single-molecule RNA in situ hybridization

RNA scope hybridization was performed with the RNA scope Multiplex Fluorescent v2-kit (Advanced Cell Diagnostics, Inc.) according to the manufacturer's instructions with the following modifications. Mice were perfused intracardially with artificial cerebral spinal fluid, the dissected brains postfixed with 4% paraformaldehyde for 2 hours at 4°C, and subsequently cryoprotected in 30% sucrose in PBS for 24 hours at 4°C and

frozen at -80°C for up to 3 months. The brains were cut coronally at 14 μm with a cryotome, mounted on a SuperFrost glass slide (Thermo Fisher Scientific), and stored at -80°C until use. Tissue sections were then dried in the ACD hybridizer at 37°C for at least 1 hour, treated with hydrogen peroxide for 10 min, and dried before protease treatment at 60°C for 30 min. The boiling step in the antigen-retrieval procedure has been omitted and the sections were digested in Protease Plus (Advanced Cell Diagnostics, Inc.) solution for 15 min at 40°C. Each RNA signal has been developed sequentially by specifically targeting each probe with horseradish peroxidase, which converted fluorescently labeled tyramide (TSA Plus fluorescein, Cy3 or Cy5 kit, PerkinElmer) into an insoluble stain around the RNA of interest. The final concentration of tyramide in TSA buffer solution was 1:1500. Custom RNAscope target probes (all targeting mouse transcripts) were purchased from Advanced Cell Diagnostics (*Slc17a7*, *Lingo1*, and *Cry1*), as well as standard RNAscope positive (housekeeping genes: *Polr2a*, *PPIB* and *Ubc*) and negative (bacterial housekeeping gene: *DapB*) control probes.

For imaging, a confocal laser-scanning microscope (LSM 710, Carl Zeiss, ZEN imaging software) was used. The images were acquired using a 40 $\times$ (numerical aperture 1.4) objective and a pinhole set at 1 airy unit, pixel dwell time 3.15 μs , and laser power 1.2 to 2%. The images spanned the whole thickness of the brain slices (10 to 12 μm) in 1- μm steps and were analyzed as a maximum-intensity projection across z -stacks.

To reliably quantify mRNA dots in different brain regions, a custom Python script was written using the ImageJ image-processing framework. The script can be used as a plugin and is openly available on a GitHub repository (<https://github.com/dcolam/Cluster-Analysis-Plugin>). Images were binarized and segmented to separate nuclei-rich regions (pyramidal cell layer of the hippocampus and cell nuclei in cortex), and particle analysis was done using ImageJ. For representation purposes, mRNA dots were enlarged to improve visualization.

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SUPPLEMENTARY MATERIALS

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References

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RESEARCH ARTICLE SUMMARY

NEUROSCIENCE

Sleep-wake cycles drive daily dynamics of synaptic phosphorylation

Franziska Brüning*, Sara B. Noya*, Tanja Bange, Stella Koutsouli, Jan D. Rudolph, Shiva K. Tyagarajan, Jürgen Cox, Matthias Mann, Steven A. Brown†, Maria S. Robles‡

INTRODUCTION: Globally, circadian clock-driven protein phosphorylation is a key mechanism to temporally compartmentalize biological processes across the day in peripheral tissues. In the brain, phosphorylation of several proteins has been reported to correlate with sleep pressure, itself regulated in a circadian fashion. Locally in neurons, phosphorylation plays a critical role in the regulation of synaptic function by allowing rapid modulation of protein activity and could thus dynamically scale synaptic strength in response to circadian or sleep-driven cues. Understanding the magnitude and origin of phosphorylation dynamics within synaptic proteins on a system level would be of great value to mechanistically assess synaptic function deficiencies and to understand temporal contributions to brain pathologies.

RATIONALE: Little is known about whether and how global phosphorylation signaling in synapses is shaped in a time-dependent manner. To comprehensively characterize phosphorylation rhythms in the synaptic compartment driven by circadian and sleep-wake-dependent cues, we biochemically iso-

lated synaptoneurosomes from mouse forebrain and analyzed them with advanced mass spectrometry-based proteomics.

RESULTS: Of more than 8000 phosphopeptides in almost 2000 proteins accurately quantified in the synaptoneurosomes compartment across 24 hours, 30% oscillated in abundance. The phases of rhythmic phosphopeptides were distributed in two major clusters, corresponding to the transition from wake to sleep at dawn and sleep to wake at dusk. In addition to important synaptic constituents such as ion channels, receptors, and scaffolds, a large number of kinases were among the synaptic proteins modulated by phosphorylation in a time-dependent manner. More than half of the detected phosphorylated kinases in synapses show rhythmic phosphorylation at one or more residues. Predictive and experimental data demonstrate that widespread dynamic regulation of kinase activity is a core phospho-dependent functional process at synapses. Together, our data uncover molecular processes in synapses whose activity is temporally gated by phosphorylation, such as synaptic inhibition at dawn and excita-

tion at dusk. We further assessed circadian- and sleep-driven signals by interfering with the sleep-wake cycle by applying 4 hours of sleep deprivation at different times of the day. Sleep deprivation resulted in the loss of 98% of rhythmic phosphorylation in fore-

brain synapses but left circadian cycles unaffected in a core of 41 phosphoproteins that function in synaptic transport and scaffolding.

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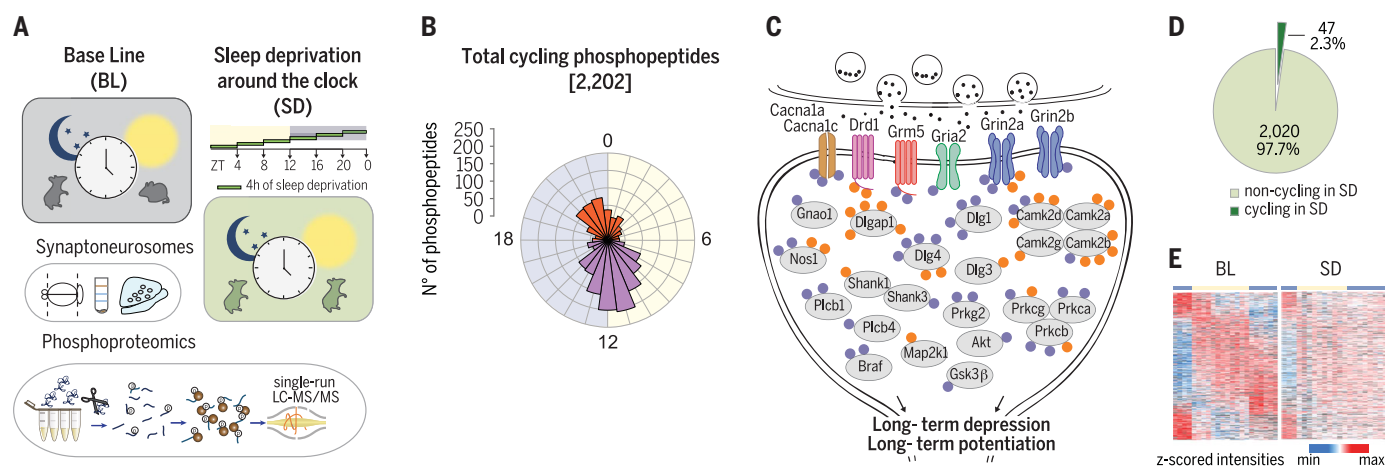
CONCLUSION: This global rhythmic phosphoproteome of isolated synaptoneurosomes reveals a major reorganization of the synaptic molecular compartment concomitant with changes in activity. Our results indicate that phospho-dependent activation of kinases in response to sleep and wake is a core driver of these synaptic phosphorylation dynamics. Together, our data point toward an association of synaptic potentiation with wakefulness (activity) and down-scaling with sleep (rest). High sleep pressure induced through sleep deprivation almost completely abrogates both peaks of daily phosphorylation cycles in synapses. We thus hypothesize that modulation of phosphorylation-mediated synaptic signaling could be a key driver underlying sleep- and wake-dependent mechanisms to regulate synaptic homeostasis and function. ■

The list of author affiliations is available in the full article online.

*These authors contributed equally to this work.

†Corresponding author. Email: charo.robles@med.uni-muenchen.de (M.S.R.); steven.brown@pharma.uzh.ch (S.A.B.)

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Daily dynamics of global phosphorylation in forebrain synaptoneurosomes under basal and sleep-deprived conditions. (A) Quantitative phosphoproteomics analyses of isolated synaptoneurosomes from rested and sleep-deprived mice around the clock. (B and C) More than 30% of phosphorylations in many synaptic components and numerous kinases cycle daily with peaks at the sleep-wake-sleep transitions. (D and E) Sleep deprivation abolished 98% of all phosphorylation cycles in synaptoneurosomes.

RESEARCH ARTICLE

NEUROSCIENCE

Sleep-wake cycles drive daily dynamics of synaptic phosphorylation

Franziska Brüning^{1,2*}, Sara B. Noya^{3*}, Tanja Bange¹, Stella Koutsouli¹, Jan D. Rudolph⁴, Shiva K. Tyagarajan³, Jürgen Cox⁴, Matthias Mann^{2,5}, Steven A. Brown^{3,†}, Maria S. Robles^{1,†}

The circadian clock drives daily changes of physiology, including sleep-wake cycles, through regulation of transcription, protein abundance, and function. Circadian phosphorylation controls cellular processes in peripheral organs, but little is known about its role in brain function and synaptic activity. We applied advanced quantitative phosphoproteomics to mouse forebrain synaptoneurosomes isolated across 24 hours, accurately quantifying almost 8000 phosphopeptides. Half of the synaptic phosphoproteins, including numerous kinases, had large-amplitude rhythms peaking at rest-activity and activity-rest transitions. Bioinformatic analyses revealed global temporal control of synaptic function through phosphorylation, including synaptic transmission, cytoskeleton reorganization, and excitatory/inhibitory balance. Sleep deprivation abolished 98% of all phosphorylation cycles in synaptoneurosomes, indicating that sleep-wake cycles rather than circadian signals are main drivers of synaptic phosphorylation, responding to both sleep and wake pressures.

Circadian clocks are endogenous oscillators present in virtually every mammalian cell. The molecular mechanism of the clock drives cycles of transcription, translation, and protein activity to regulate daily changes in physiology and behavior. Mass spectrometry (MS)-based quantitative proteomics has contributed substantially to our understanding of how circadian posttranscriptional mechanisms temporally shape metabolic processes in peripheral tissues (1, 2). Circadian phosphorylation changes by far eclipse the regulation at the transcriptional and proteome levels in amplitude (3). Temporal characterization of proteome and phosphoproteome changes in the central nervous system, by contrast, has been challenging because of the sensitivity, dynamic range, and throughput required to capture the regional, cellular, and synaptic heterogeneity. However, recent advances in MS in combination with spatial isolation methods allow the deep characterization of proteomes from different brain regions and cell populations (4, 5). In addition, high-throughput phosphoproteomic technologies are now suitable for the global characterization of phosphorylation signaling dynamics in different brain areas (6).

Numerous synaptic features—such as diffusion of receptors in membranes, channel conductance, or cytoskeleton remodeling—depend on fast phosphorylation-based control mechanisms. In particular, synaptic plasticity and scaling have been linked to phosphorylation of receptor, scaffolding, cytoskeletal, and other synaptic proteins (7, 8). Although quantitative phosphoproteomics has been applied to the synaptic compartment, technical limitations have so far precluded accurate quantification that would allow the precise characterization of global phosphorylation dynamics associated with synaptic function (7). It is thus unknown whether daily changes in synaptic activity are coupled to global dynamics of phosphorylation in synapses or, moreover, whether daily rhythms of phosphorylation temporally segregate synaptic processes. Two recent reports have addressed these technical limitations by either fractionating postsynaptic density (9) or by mapping whole-brain phosphoproteomics to synaptic protein annotations (10). They highlight a role for sleep pressure in driving synaptic phosphorylation changes associated with the kinase SIK3 (10) and downstream effectors of plasticity such as HOMER1a (9).

We applied state-of-the-art quantitative MS-based proteomics to characterize in vivo phosphorylation dynamics across the day in isolated synaptoneurosomes from mouse forebrain, resulting in the most comprehensive time-resolved phosphoproteome of synapses to date. In combination with in-depth proteomics (11), we found that more than one-fourth of the individual phosphorylation sites in synaptic proteins oscillate daily and independently of protein abundance. Temporally modulated

phosphorylation networks gate synaptic processes at both dawn and dusk, primarily dependent on sleep-wake cycles. Thus, maintaining sleep pressure approximately constant across the day leads to a dramatic ablation of global phosphorylation cycles, suggesting a dominant role of both sleep and wake pressure in synaptic phosphorylation dynamics. In turn, our analyses suggest that these dynamics profoundly shape both synaptic function and downstream regulatory networks.

Results

In-depth phosphoproteomic profiling of synaptoneurosomes

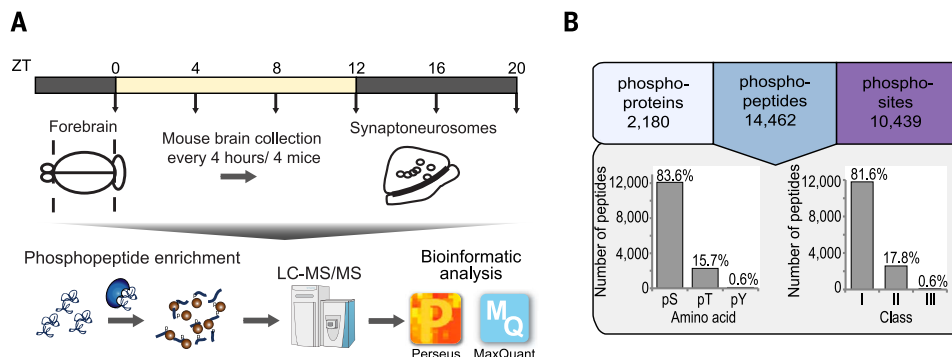
To characterize daily dynamics of phosphorylation abundance specifically in synapses, we biochemically isolated synaptoneurosomes from mouse forebrains (11). Mice were kept in 12-hour:12-hour (light:dark) schedules and then euthanized in biological quadruplicates at six time points, every 4 hours, across 24 hours ($n = 24$ mice). We used a rapid method based on Percoll gradients to prepare synaptoneurosomes from forebrains, containing both pre- and postsynaptic components (12), and immediately flash-froze them to prevent dephosphorylation. To achieve sufficient throughput for our time-dependent experiments, we used the EasyPhos method (13) to enrich phosphopeptides from only 1 mg of protein homogenate for each synaptoneurosome preparation. The MS-based quantitative phosphoproteomics workflow, consisting of single runs on a high-resolution, high-sensitivity quadrupole-Orbitrap HF-X mass spectrometer, is shown in Fig. 1A. Across all samples, this resulted in a total of 10,439 unique phosphosites identified in 14,462 phosphopeptides, mapping to more than 2000 proteins (Fig. 1B and table S1). Comparing phosphorylated amino acids in synapses with our previous circadian study in the liver (3) revealed similar proportions [83.6% phosphoserine (pS), 15.7% phosphothreonine (pT), 0.6% phosphotyrosine (pY)] (Fig. 1B). Phosphopeptide intensities between measurements were highly reproducible in both biological replicates and time points [mean Pearson correlation coefficient (r) = 0.88 and 0.83, respectively] (fig. S1A). The intensities of phosphopeptides in synaptoneurosomes ranged over five orders of magnitude (fig. S1B), similar to what we found in liver, indicating that the synaptic compartment still has a wide quantitative range of phosphorylation levels. To indirectly evaluate our isolation method, we performed a Fisher's exact test on the total phosphoproteome dataset. Of all annotated protein keywords, the top two most significant are "synapse" and "cell junction" ($P < 10^{-40}$ for both), and the other highly enriched ones are also relevant to synaptic function, even when analyzing every time point separately (fig. S1, C to E, and materials and methods). Our data

¹Institute of Medical Psychology, Faculty of Medicine, LMU Munich, Germany. ²Department of Proteomics and Signal Transduction, Max-Planck Institute of Biochemistry, 82152 Martinsried, Germany. ³Institute of Pharmacology and Toxicology, University of Zurich, Zurich, Switzerland. ⁴Computational Systems Biochemistry, Max-Planck Institute of Biochemistry, Martinsried, Germany. ⁵Novo Nordisk Foundation Center for Protein Research, Faculty of Health Science, University of Copenhagen, Copenhagen, Denmark.

*These authors contributed equally to this work.
†Corresponding author. Email: charo.robles@med.uni-muenchen.de (M.S.R.); steven.brown@pharma.uzh.ch (S.A.B.)

Fig. 1. Phosphoproteome characterization of synaptoneurosomes isolated across the day from mouse forebrains. (A) Experimental workflow. (B) Number of identified phosphoproteins, phosphopeptides, and phosphosites in all measured samples.

(Bottom left) Distribution of phosphorylated amino acids [serine (pS), threonine (pT), and tyrosine (pY)]. (Bottom right) Number of phosphorylated residues from different classes according to localization probability: class I (probability > 75%), class II (probability = 50 to 75%), and class III (probability < 50%).



show the power of combining high-throughput phosphoproteomics with biochemical isolation of synaptoneurosomes to deeply profile phosphorylation in this discrete neuronal compartment.

Daily rhythms of the synaptic phosphoproteome

Next, we performed statistical cycling analysis in the circadian module of the Perseus software (2, 3, 14) to filter and cosine-fit the phosphopeptide intensities. A total of 2202 (30.4%) of the 7257 phosphopeptides accurately quantified in at least 50% of the samples oscillate in abundance with a rhythm of 24 hours ($q < 0.05$) (Fig. 2, A and B; table S2; and materials and methods). We detected rhythmic phosphorylations in more than half of the synaptic phosphoproteins (838 out of the total 1655) (Fig. 2C). These cycling sites were localized with high probability to a single residue (mean 0.97), and their amino acid distribution was similar to that of the total dataset (Fig. 1B and fig. S2A). Cycling phosphopeptides had an intensity distribution similar to that of the total phosphoproteome (fig. S2B), implying that circadian phosphoregulation is not biased by abundance. Little is known about the magnitude of dynamic phosphorylation changes in the synaptic compartment, and our data revealed that these changes are substantial: The mean amplitude changes are more than threefold, with hundreds of sites at more than 10-fold (Fig. 2D).

To assess the extent to which these phosphorylation dynamics depend on protein abundance changes, we quantitatively compared the levels of phosphopeptides with the abundance of the corresponding protein. Almost 90% of proteins with cycling phosphopeptides were quantified at the protein level in our companion study (11), and of these, only 5% significantly oscillate in abundance ($q < 0.05$, period = 24 hours) (Fig. 2E). Even the small fraction of rhythmic proteins with oscillating phosphorylation displays different phases across the day, and furthermore, multiple sites in the same protein generally behaved differently (Fig. 2F and fig. S2C). In

the minor population of rhythmic proteins carrying cycling phosphorylation, the mean amplitudes at the phosphorylation level were 10-fold larger than those at the protein level (Fig. 2G). Our data clearly establish that protein phosphorylation in forebrain synaptoneurosomes is highly dynamic across the day and almost completely independent of protein abundance, suggesting another layer of synaptic functional regulation.

Temporal compartmentalization of synaptic protein phosphorylation

Our previous study in liver revealed that dynamic phosphorylation drives daily organ functions to a previously unappreciated degree (3). Examining the phosphorylation rhythms in the synaptic compartment showed that the phases of rhythmic phosphopeptides gathered in two distinct clusters. The larger one, at the light-to-dark transition when mice start to be active, contains two-thirds of the phosphopeptides, whereas the remaining ones peak at the end of the night, preceding the sleep phase (Fig. 3A). This phase distribution indicates a major rewiring of protein phosphorylation and presumably synaptic function at the wake-to-sleep and sleep-to-wake transitions. In order to identify synaptic functions that are temporally compartmentalized by protein phosphorylation, we searched for statistically enriched protein annotations in each of the two defined phase clusters (Fisher's exact test, $P < 0.05$) (materials and methods). At the end of the resting phase, our analysis found keywords corresponding to "cell adhesion" and "cell junction" as well as "ion channels," "ion transporters," "hydrolases," and "kinases highly enriched." By contrast, "cell projection," "cytoskeleton," and "ubiquitin conjugation protein" annotations are overrepresented in the phosphopeptide cluster at the end of the activity phase (Fig. 3, B and C, and table S3). Proteins involved in cell division and mitosis were also enriched in the phosphorylation cluster at the end of the activity phase, likely because several cell cycle kinases, such as CDK5, are also important for synaptic activity (15). Thus, our

results imply that different remodeling processes that are known to occur at synapses (16) might occur separately in temporally distinct nodes.

Synapses are hubs of kinases

We next focused our attention on the major and specific enrichment of kinases, key regulators of almost all cellular processes, in the phosphopeptide cluster that peaks at the end of the resting phase. We identified almost 500 phosphorylated peptides from a total of 128 kinases from all major families in mammals. Thus, a fifth of the total mouse kinome is not only present in the synaptic compartment but also detectable in a phosphorylated form (fig. S3A). More than half of these kinases show at least one rhythmic phosphorylation ($q < 0.05$) (Fig. 4, A and B, and table S4), and these belong to all major kinase families, with a higher representation of AGC threonine/serine kinases (Fig. 4, C and D). All of the 66 kinases with rhythmic phosphorylation were also quantified at the protein level in the synaptic compartment (11); however, only four of them cycled in protein abundance (fig. S3B). Therefore, phosphorylation, rather than protein abundance, likely regulates temporal kinase function at synapses across the day. Our analyses also detected cycles of phosphorylation and protein abundance in several phosphatases at the synapses but in a lesser extent compared with kinases. Out of the 77 phosphatases detected in synapses, only three cycle at the protein level, and 10 out of 20 phosphorylated phosphatases showed rhythms of phosphorylation (fig. S4, A to D).

The overall phase distribution of rhythmic phosphopeptides from kinases resembles the total cycling synaptic phosphoproteome (Figs. 3A and 5A), suggesting that phosphorylation-dependent temporal activation of kinases contributes to the global phosphorylation rhythms in synapses. However, because site-specific phosphorylation does not always imply changes in kinase activity, we next set out to identify temporally activated kinases with an unbiased workflow that uses high-confidence protein-

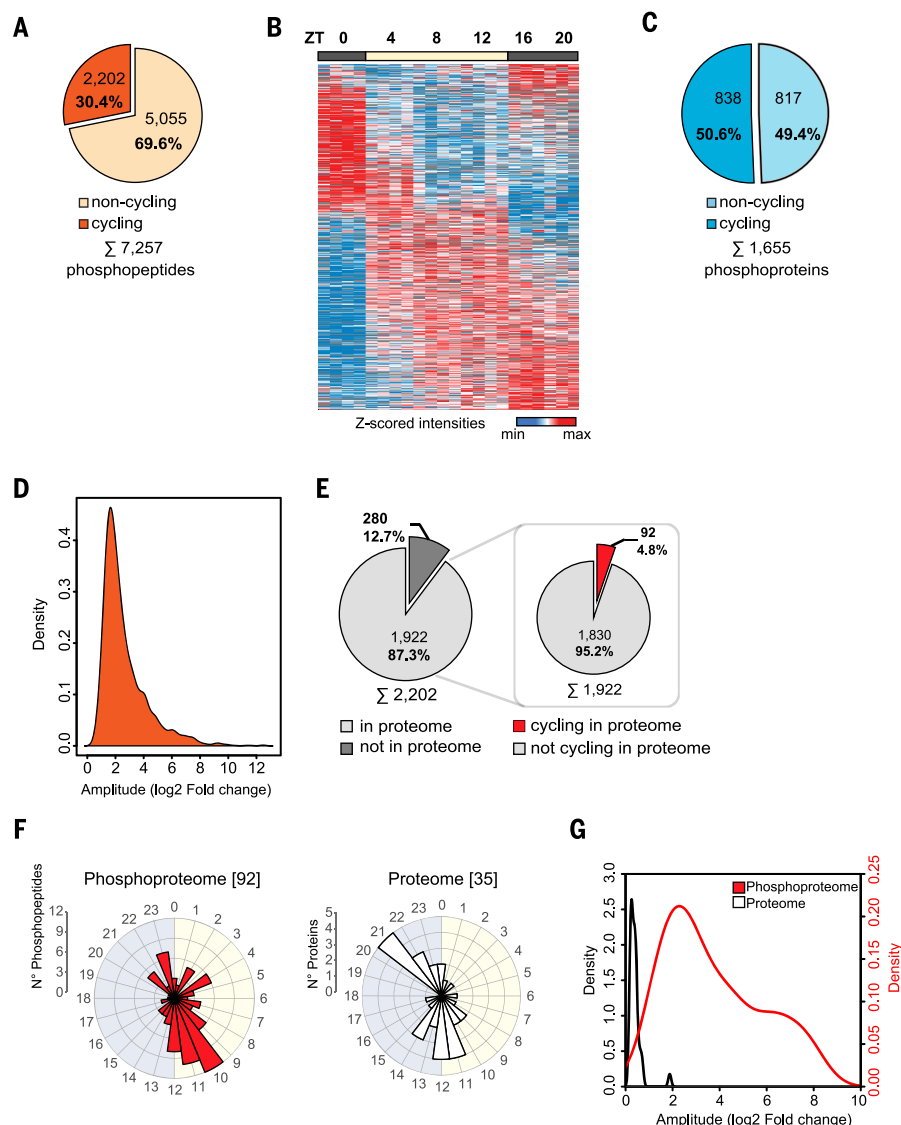


Fig. 2. Daily rhythms of the synaptic phosphoproteome. (A) Pie chart showing the percentage of phosphopeptides oscillating ($q < 0.05$, period = 24 hours) in synaptoneurosomes. (B) Heat map with the intensities (log2 z-scored normalized) of each cycling phosphopeptide (rows) across the measure samples (columns) ordered by peak of abundance. (C) Pie chart with the percentage of proteins carrying at least one cycling phosphorylation in synaptoneurosomes. (D) Density plot showing the calculated amplitudes of rhythmic phosphopeptides in synaptoneurosomes. (E) Pie charts showing (left) the percentage of cycling phosphopeptides from proteins quantified in our proteome study and (right) the fraction of cycling phosphopeptides in proteins that are also rhythmic at the protein level. (F) Rose plots representing the phase distribution of rhythmic phosphopeptides (left) and their corresponding oscillating proteins (right). (G) Density plots comparing the amplitudes of the rhythmic phosphopeptides and the corresponding oscillating proteins.

protein interaction networks and large-scale phosphorylation data to retrieve protein signaling functionality. This PHOTON pipeline assigns a score to each protein according to the phosphorylation status of their interacting proteins (17). Because these scores reflect the changes in phosphorylation levels of their substrates and interactors, kinases that are activated rather than only phosphorylated should have PHOTON scores that cycle across the 24 hours, with the maximum score indi-

cating the peak of kinase activity. From the 66 synaptic kinases with rhythmic phosphorylation, this analysis resulted in rhythmic activity patterns for 13 of them (materials and methods). Of these, 11 are active at the sleep-wake transition, including protein kinase C (PRKCA, PRKCB, and PRKCG) and Ca^{2+} /calmodulin-dependent kinase 2 (CAMK2B and CAMK2G). Conversely, the tyrosine-protein kinase ABL2 and the serine/threonine-protein kinase DCLK1 showed the opposite behavior, peaking in

activity at the wake-sleep transition (Fig. 5, B and C).

To substantiate the PHOTON prediction data, we used a second computational method [kinase substrate enrichment analysis (KSEA)] that infers kinase activity by using curated kinase-substrate relationships (18). This second method estimated, very similarly to PHOTON, the activation of PKC and CAMK2 at the sleep-wake transition (fig. S5 and materials and methods). We verified these kinase activity patterns by immunoblotting using phospho-specific antibodies against phosphorylated residues known to regulate the activity of these two kinases (fig. S6, A and B) (19, 20). The KSEA algorithm also identified additional putative temporally activated kinases with rhythms of phosphorylation (fig. S5). For example, this method predicted that GSK3 β , a molecular switch in synaptic activation and plasticity (21), is active during the sleep stage. Such predicted activation would be in antiphase to the phosphorylation cycle of two residues known to inhibit its kinase activity, S389 and S9 (22, 23). The former was detected in our data with a peak at ZT11, and the latter was further confirmed by means of immunoblotting (fig. S6C).

Considering the published literature about rhythmically regulated kinases predicted by PHOTON and KSEA, the kinases activated at the transition to the wake phase (CAMK and PKC) are associated with excitatory synaptic activity (8), whereas those activated at the transition to and during the sleep phase (ABL2, DCLK1, and GSK3 β) are associated with inhibitory synaptic activity (24, 25). An identical temporal compartmentalization of synaptic function was independently predicted by PHOTON by means of phosphorylation dynamics of Gene Ontology (GO) molecular functions (materials and methods); it inferred that the triggering of inhibitory synaptic mechanism, involving γ -aminobutyric acid (GABA), occurs at the end of the wake period, whereas glutamate-mediated synaptic excitatory activity was predominantly associated to the sleep-wake transition (fig. S7). These data are consistent with the roles of these synaptic types in sleep and wake, respectively (26, 27).

Sleep deprivation abrogates synaptic phosphorylation rhythms

We hypothesized that the sharp biphasic distribution of synaptic phosphorylation patterns at the wake-sleep and sleep-wake transitions might reflect either buildup and dissipation of sleep pressure (a sleep homeostat), or alternatively a circadian (time-of-day) mechanism, or a combination of the two. To test their relative contributions, we subjected mice to 4 hours of sleep deprivation (SD) by means of gentle handling (28) before each time point

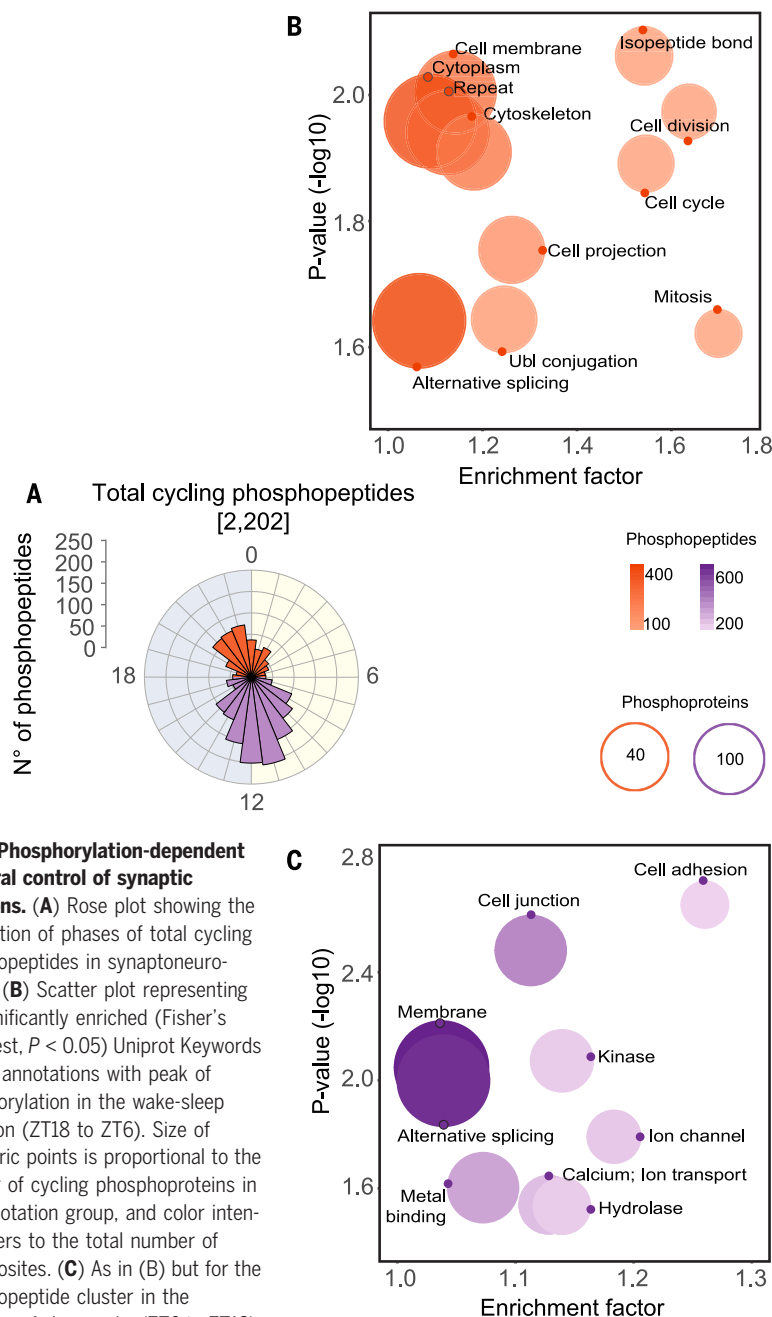


Fig. 3. Phosphorylation-dependent temporal control of synaptic functions. (A) Rose plot showing the distribution of phases of total cycling phosphopeptides in synaptoneurosome. (B) Scatter plot representing the significantly enriched (Fisher's exact test, $P < 0.05$) Uniprot Keywords protein annotations with peak of phosphorylation in the wake-sleep transition (ZT18 to ZT6). Size of geometric points is proportional to the number of cycling phosphoproteins in the annotation group, and color intensity refers to the total number of phosphosites. (C) As in (B) but for the phosphopeptide cluster in the transition of sleep-wake (ZT6 to ZT18).

and collected brains every 4 hours in 24 hours ($n = 4$ brains per time point) (Fig. 6A). A similar protocol of SD across the 24-hour time course equalizes sleep pressure to keep it constantly high (29). First, we empirically verified that this was the case by measuring the amplitude of electroencephalogram (EEG) oscillations during sleep (0.5 to 4 Hz, the “delta” range proportional to sleep pressure) (30) and demonstrating that this was similar at each time point to that maximally observed spontaneously in the day before the manipulation. Although some fluctuation in delta power across time points was still observed, we estimated it to be less than a fifth of what is

observed under the same conditions across the normal circadian day. We also demonstrated that the pattern and timing of sleep and activity in the subsequent day were not altered by this protocol—that the protocol was not shifting the phase of the circadian clock (Fig. 6B and figs. S8, A and B, and S9). Next, we prepared synaptoneurosome from forebrains of SD mice, and as in baseline (BL) conditions, we enriched phosphopeptides before MS analysis (Fig. 6A and materials and methods). We accurately quantified 7021 phosphopeptides in at least 50% of the samples, which are very similar values to those in the BL experiment, with more than 90% (6526

phosphopeptides) overlap between them (fig. S10A). Cycling analysis of the 6526 phosphopeptides revealed almost complete abrogation of rhythmic phosphorylation in the synaptic compartment of SD mice. Only 2.3% (47 phosphopeptides corresponding to 41 proteins) of the phosphopeptides rhythmic in BL maintained the cycle in SD (period = 24 hours, $q < 0.05$) (Fig. 6, C and D; table S5; and materials and methods). These few remaining cycling phosphopeptides oscillated with similar amplitudes and with comparable phases in both conditions (Fig. 7, A to C, and table S6), suggesting that they might be driven primarily by the circadian clock. Of the corresponding 41 synaptic phosphoproteins, 31 belong to interconnected cellular structures such as cytoskeleton, synaptic scaffolding, membrane, vesicle trafficking, and ubiquitin mechanisms, all of which are important for synaptic integrity and function (fig. S11) (31–33). Aside from this small fraction, the remaining 98% of phosphorylations, affecting all aspects of synaptic biology, were severely affected by SD across the day, leading to a dramatic loss in rhythmicity (fig. S10, B to D).

Discussion

Synaptic plasticity and function dynamically change across the day (34). It is already known that changes in synaptic activity are associated with the phosphorylation of several signaling proteins (8, 33). However, our large-scale quantitative phosphoproteome of isolated synaptoneurosome resulted in a comprehensive time-resolved map of synaptic phosphorylation across the entire wake and sleep phases. The phosphoproteome is much more dynamic than the proteome (50% of synaptic proteins showing cyclic phosphorylation at one or more sites versus only 12% of synaptic proteins whose abundance oscillates). Moreover, similar to our previous finding in the liver (3), mean fold-changes of the phosphoproteome are more than threefold higher than in the proteome. At 7000 accurately quantified phosphopeptides, our analysis allowed in-depth bioinformatic analysis, retrieving both known and unknown temporally regulated processes at synapses. Overall, the phases of cycling phosphorylation fall into two main clusters just preceding daily activity-rest transitions, when the mouse typically wakes up or falls asleep, implying a major role of synaptic phosphorylation in regulating these transitions. This pattern contrasts markedly with that of the rhythmic phosphoproteome of total forebrain (including entire cells, not just synapses), where phosphorylation peaks gather at the wake and sleep periods (fig. S12), similarly to what we observed for the total rhythmic forebrain proteome described in (11).

Numerous kinases are expressed in the brain, and some of them also have been localized

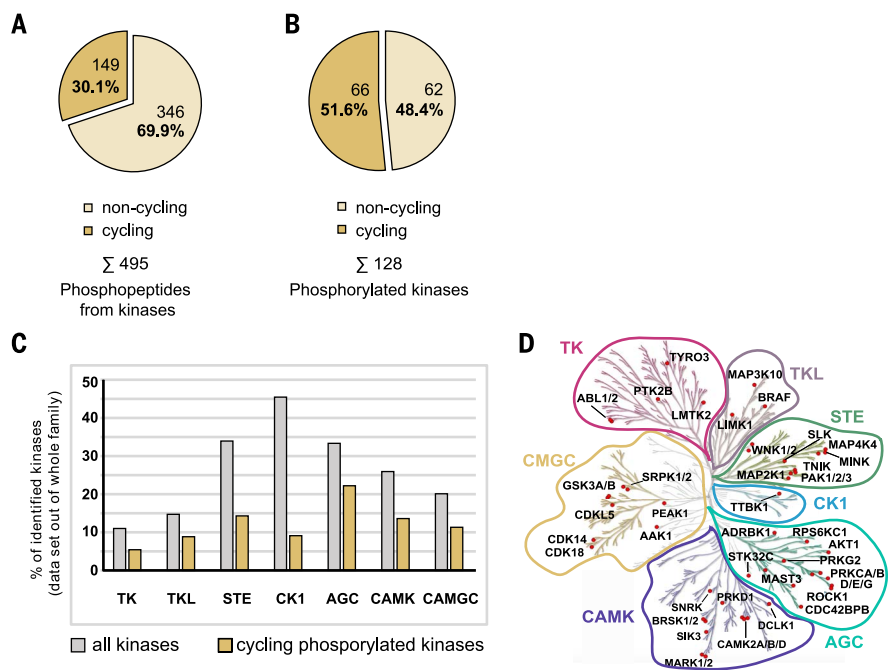


Fig. 4. Synapses are hubs of kinases. (A) Pie chart showing the percentage of cycling and not cycling phosphopeptides from kinases. (B) Pie chart showing the percentage of kinases with at least one cycling and noncycling phosphorylation. (C) Bar plot representing the percentage of all (gray) and cycling (orange) phosphorylated kinases quantified in synaptoneurosomes from each of the major mammalian kinase families. (D) Kinases with at least one cycling phosphorylation annotated to the major kinase families (52 out of the total 66 cycling) using www.kinhub.org. TK, tyrosine kinases; TKL, tyrosine kinase-like; STE, homologs of the yeast *STE7*, *STE11*, and *STE20* genes; CK1, casein/cell kinase 1 family; AGC, protein kinase A, G, C families; CAMK, calmodulin/calcium-regulated kinases and some non-calcium-regulated families; and CMGC is CDK, MAPK, GSK3, and CLK kinase families. Atypical kinases are not shown.

to the synaptic compartment (35). However, our study clearly identifies the synapse as a major kinase hub. We detected more than 100 phosphorylated kinases (20% of the total kinome) and found that 50% show daily rhythmic phosphorylation of least one residue. Combining protein interaction network data with our quantitative phosphoproteome revealed that these phosphorylation changes regulate the activity of at least a subset of them, predictions that we verified through immunoblotting of known activating and inhibiting phosphoresidues for some kinases. Prediction and empirical validation of kinase activities notably matched to the phosphorylation profiles of known substrates detected in our dataset (fig. S6). Although the existing network data are sufficient to associate rhythmic activity with a substantial subset of the kinases, more complete networks and meta-analyses will likely establish activity changes for a much larger fraction of the cycling synaptic phosphoproteome in the future. Together, the combination of predictive and experimental data places extensive temporal regulation of kinase activity as a core phospho-dependent functional process at the synapse.

An obvious question leading from these data concerns the regulatory target of this con-

trol. Considering our predictive and experimental data, we speculate that one possible consequence could be temporally distinct windows for promotion of synaptic long-term potentiation (LTP) and long-term depression (LTD). Kinases with higher activity in anticipation of the wake period are involved in LTP, such as CAMK2 and PRKC, which function directly downstream of *N*-methyl-D-aspartate receptor (NMDAR) Ca^{2+} signaling (8). By contrast, ABL2 and DCLK1, two kinases that modulate structural synaptic plasticity (24, 25), peak in activity in anticipation of sleep. Lack of ABL2 leads to elevated NMDAR synaptic currents (25); therefore, ABL2 activation at the beginning of the resting phase may mediate synaptic down-scaling. Moreover, phospho-dependent activation of GSK3 β during sleep regulates LTD and GABA receptor (GABAR) trafficking (21), and its activity is in turn blocked at the wake transition by LTP-mediated phosphorylation of its inhibitory site S9 (36) and S389. This mechanistic speculation is further supported by the extensive overlap of phosphorylated residues increased by LTP induction in mouse hippocampus (33) with those peaking at the transition to the wake phase in our dataset (fig. S13). Together, our data point toward an association of synaptic potentiation

with wakefulness (activity) and synaptic down-scaling with sleep (rest). This supports the general synaptic homeostasis hypothesis for sleep—that synaptic down-scaling is a key function of sleep (37) at the molecular level—and provides starting points for a multitude of mechanistic investigations.

Critically, this functional support is created both by phosphorylation rhythms dependent on the sleep-wake cycle and their peaks clustering at the wake-sleep-wake transitions. Thus, an experimental protocol to produce constant sleep pressure across the 24-hour day almost entirely abrogates global diurnal oscillation of phosphorylation in the synaptic compartment. This discovery establishes the importance of the homeostatic regulation of sleep over any other mechanism in generating daily rhythms of phosphorylation to modulate synaptic function. However, these phosphorylation changes are governed both by wake and by sleep. A recent analysis of wake-dependent protein phosphorylation in total mouse brain showed that increasing hours of SD resulted in increasing average phosphorylation levels of 80 synaptic proteins (10). We examined our data and found phosphorylations in all of those 80 proteins, with sites showing increased phosphorylation at times of high sleep pressure in 69 of them. However, our amino acid-resolution data reveal a more nuanced picture than a simple overall increase in phosphorylation, with both sleep- and wake-dependent phosphorylation at different residues in these proteins. Another recent phosphoproteomics study in the synaptic compartment showed that sleep regulates dephosphorylation of AMPA receptors in response to HOMER1a-dependent immediate early transcriptional signaling (9). Our data independently support these findings, extend them to several other neurotransmitter receptors, and supply their diurnal rhythms of phosphorylation in response to sleep and wake pressure. Globally across the entire phosphoproteome, we found about one-third of cycling phosphorylations to be maximal at the end of the wake phase when sleep pressure is highest and two-thirds to peak at the end of the sleep phase before waking.

Although the vast majority of protein phosphorylation appears to be regulated by sleep or wake states, a small number of rhythmic phosphorylations remain unchanged in amplitude and phase under SD. These phosphorylations occur in a highly connected node of proteins involved in both synaptic vesicle trafficking (molecular motors and microtubule-associated proteins) and synaptic scaffold (SHANK3, PICCOLO, and BASSOON). Circadian regulation of cortical function is already well documented at both behavioral and molecular levels (38). Our data imply that even as sleep-wake pressures dynamically reconfigure

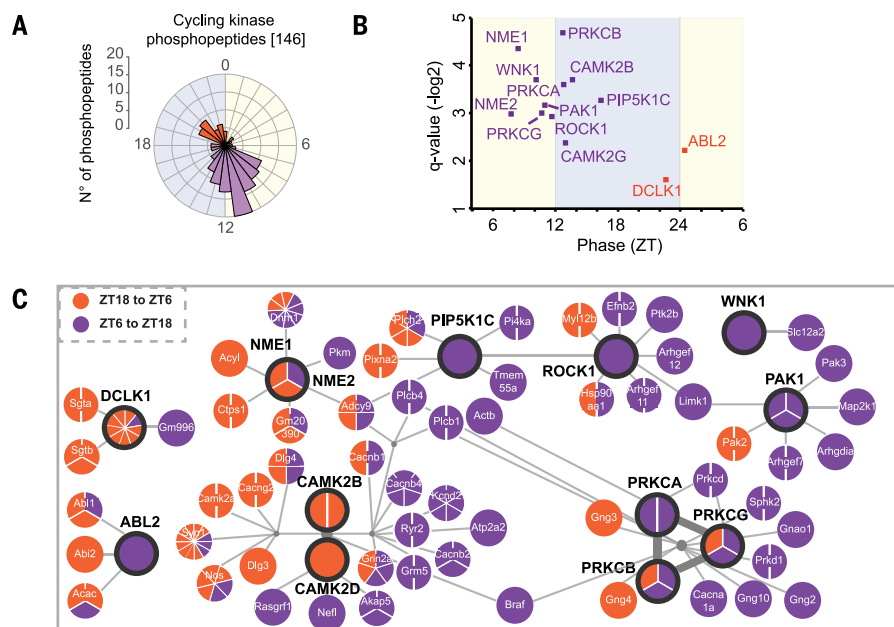


Fig. 5. Rhythmic phosphorylation and activation of synaptic kinases. (A) Rose plot with the phases of cycling phosphopeptides from kinases detected in synaptoneurosomes. Colors denote the clusters of wake-sleep transition (ZT18-ZT6, orange) and sleep-wake transition (ZT6-ZT18, purple). (B) Scatter plot showing kinases temporally activated predicted by PHOTON. The x axis indicates the phase of activation, and the y axis indicates the *q* value of the cycling analysis by using PHOTON scores. Kinase names are color-coded according to the two phosphopeptide clusters shown in (A). (C) Protein interaction network of PHOTON predicted cycling active kinases ($q < 0.05$) with rhythmic phosphorylations with their cycling phosphorylated interactors. Nodes are divided on the basis of the number of cycling phosphorylations identified in each protein and color-coded according to the clusters in (A). Nodes with thick lines denote those kinases with rhythmic phosphorylated residues that are also predicted to be temporally activated by PHOTON.

synaptic structure and function, circadian control—or at least circadian clock-sleep interactions—continue to influence cortical function. For example, in our accompanying manuscript, we show that synaptic provisioning of RNA can be independently controlled by the circadian clock or by sleep-circadian interactions (17). For both synaptic protein abundance and synaptic protein phosphorylation, however, by far the major diurnal influence is that of daily sleep and wake. The mechanism of this influence remains unknown and could include not only direct signaling (such as receptor-driven kinase cascades) but also indirect effects such as light-dark cycles or changes in cortisol or body temperature, which have been shown to play roles in sleep-wake-dependent gene expression (39) and in circadian entrainment (40, 41).

This study presents *in vivo* proof of rhythmic orchestration for thousands of phosphorylation events in synapses across the day. Our study demonstrates a central role for sleep-wake cycles in phosphorylation-dependent regulation of many aspects of synaptic function in response to both sleep and wake pressure, potentially modulating processes that range from plasticity to cellular house-

keeping. Interfering with rest-activity cycles almost completely abolished rhythms of phosphorylation in synaptic proteins. Our findings may thus contribute to understanding the molecular basis of mental dysfunction frequently associated with SD.

Materials and methods

Animals and tissue collection

All experiments were performed in accordance with the Animal Welfare Officer of the University of Zürich and the veterinary authorities of the Canton of Zürich. For the circadian base line (BL) experiments, 10-week-old male C57BL/6 mice were housed with free access to food and water and entrained to a 12-hour/12-hour light-dark schedule for 14 days. Mice were sacrificed at 4-hour intervals over 1 day. At the time points overlapping with light transitions (ZT0 and ZT12), euthanasia was performed by cervical dislocation before the light change. For the around-the-clock SD, mice were allowed to acclimatize to a 12-hour light/12-hour dark cycle for 14 days. Six groups of mice were sleep deprived for 4 hours by gentle handling (cage exchange and introduction of novel objects) before being sacrificed at different times of day (ZT0, ZT4,

ZT8, ZT12, ZT16, and ZT20). Animals were sacrificed by cervical dislocation avoiding anesthetics, which are potent inhibitors of synaptic transmission. In addition, to boost preservation of phosphorylation, total preparation time was minimized, and samples were kept cooled at every step of the protocol.

Electroencephalogram (EEG) recording and sleep data analysis

Adult wild-type (WT) mice were used for surgery (8 to 10 weeks old at surgery). Mice were implanted epidurally under isoflurane anesthesia for EEG recording. Right before and 24 hours after surgery mice were treated with an analgesic (Temgesic, 0.1 mg/kg, intraperitoneal). Gold-plated miniature screws (0.9 mm diameter) were used as EEG electrodes and positioned on the left hemisphere above the frontal cortex (1.5 mm anterior to bregma, 1.5 mm lateral to the midline) and the parietal cortex (2 mm posterior to bregma and 2 mm lateral to the midline). The reference electrode was placed above the cerebellum (2 mm posterior to lambda, 0 mm lateral to the midline). Screws were connected to copper wires and fixed to the skull with dental cement (Paladur 2-component system). Electromyography (EMG) was recorded using two gold wires (0.2 mm diameter) inserted bilaterally in the neck muscle. After 1 week of recovery EEG-EMG was recorded continuously for 7 days. 2 cohorts of 6 and 8 mice, respectively, underwent a BL days recording and 3 SD days with 48 hours of recovery in between. Cohort 1 underwent SD at ZT4-8, ZT12-16 and ZT16-20. Cohort 2 underwent SD at ZT0-4, ZT8-12 and ZT20-24. SD was performed by gentle handling as described by (42). Both EEG and EMG signals were amplified (factor 2000), analog filtered (high-pass filter: -3 dB at 0.016 Hz; low-pass filter: -3 dB at 40 Hz, less than -35 dB at 128 Hz), sampled with 512 Hz, digitally filtered (EEG: low-pass FIR filter 25 Hz; EMG: band-pass FIR 20-50 Hz) and stored with a 128 Hz resolution. EEG power spectra were computed for 4-s epochs by a Fast Fourier Transform routine within the frequency range of 0.5 to 25 Hz. Between 0.5 Hz and 5 Hz, 0.5 Hz bins were used, and between 5 and 25 Hz 1 Hz bins were used. The corresponding slow-wave-activity (SWA) was calculated using the raw parietal and frontal EEG, as well as the raw and integrated EMG to visually score three vigilance states [non-rapid eye-movement sleep (NREM) sleep, rapid eye-movement sleep (REM), and wake] for 4-s epochs. Epochs containing artifacts were identified and excluded from the spectral analysis. Data analysis was carried out using MATLAB version R2015a (The Math Works, Natick, MA, USA). Relative frontal SWA was calculated relative to the mean SWA at ZT8-12 during the BL day. Sleep loss was calculated

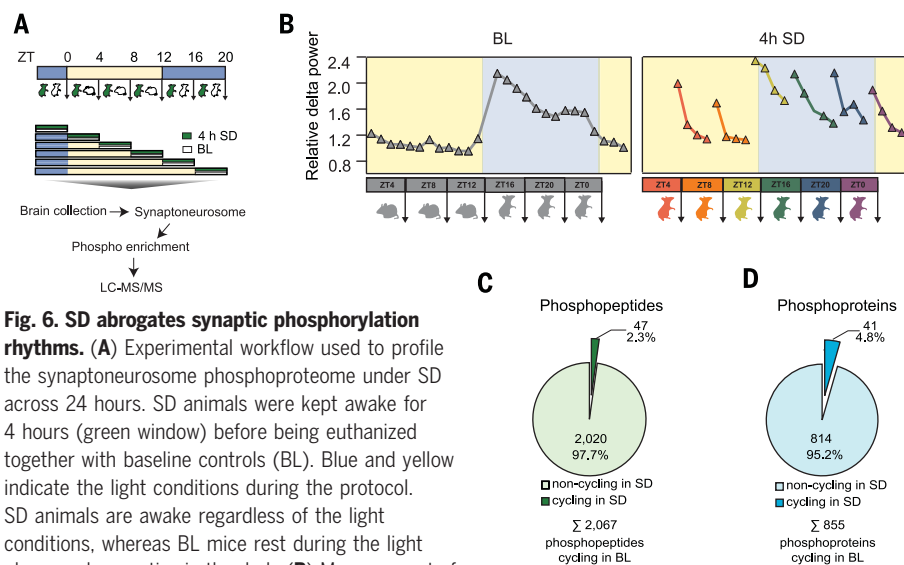


Fig. 6. SD abrogates synaptic phosphorylation rhythms.

(A) Experimental workflow used to profile the synaptoneurosome phosphoproteome under SD across 24 hours. SD animals were kept awake for 4 hours (green window) before being euthanized together with baseline controls (BL). Blue and yellow indicate the light conditions during the protocol. SD animals are awake regardless of the light conditions, whereas BL mice rest during the light phase and are active in the dark. (B) Measurement of the EEG of relative frontal delta power during NREM sleep (triangles, percent of the last 4 hours of the baseline light periods) for the same time courses in BL (left) and SD (right). (C) Pie chart indicates the fraction of cycling phosphopeptides (period = 24 hours, $q < 0.05$) in forebrain synaptoneurosomes from SD mice out of the 2067 rhythmic in BL mice. (D) Pie chart shows the fraction of proteins with at least one cycling (period = 24 hours, $q < 0.05$) phosphopeptide in synaptoneurosomes of SD mice out of the 855 oscillating under BL conditions.

by comparing NREM sleep amount in each 4h SD slot to the sleep amount in the same time of day of the corresponding BL day [1-way analysis of variance (ANOVA), $P < 0.05$]. Sleep latency was analyzed by measuring the time each mouse stayed awake after the end of each 4 hours SD until it slept for more than 1 min (1-way ANOVA, $P < 0.05$).

Purification of synaptoneurosomes

Synaptoneurosomes and total mouse forebrain samples were prepared as described previously (12). In brief, brain was isolated and rapidly cooled to 4°C, washed in ice-cooled sucrose buffer (320 mM Sucrose, 5 mM HEPES, pH 7.4) followed by homogenization with a Teflon-glass tissue grinder using a motor-driven pestle keeping samples cooled. To isolate synaptoneurosomes, each forebrain homogenate was centrifuged at 1000 g for 10 min. 2 ml of the supernatant were loaded over discontinuous Percoll gradients (3%, 10%, and 23% Percoll in sucrose buffer) and centrifuged at 31,000 g for 5 min. The fraction at the interfaces between 3-10% and 10 to 23% were collected and further centrifuged at 20,000 g to pellet synaptoneurosomes. All centrifugation steps were performed at 4°C. All solutions were supplemented with complete protease inhibitor cocktail (Roche), 0.05 mM DTT, 0.1 mM PMSF and RNaseOUT 20 U/10 µl (Invitrogen).

Sample preparation and phosphopeptides enrichment

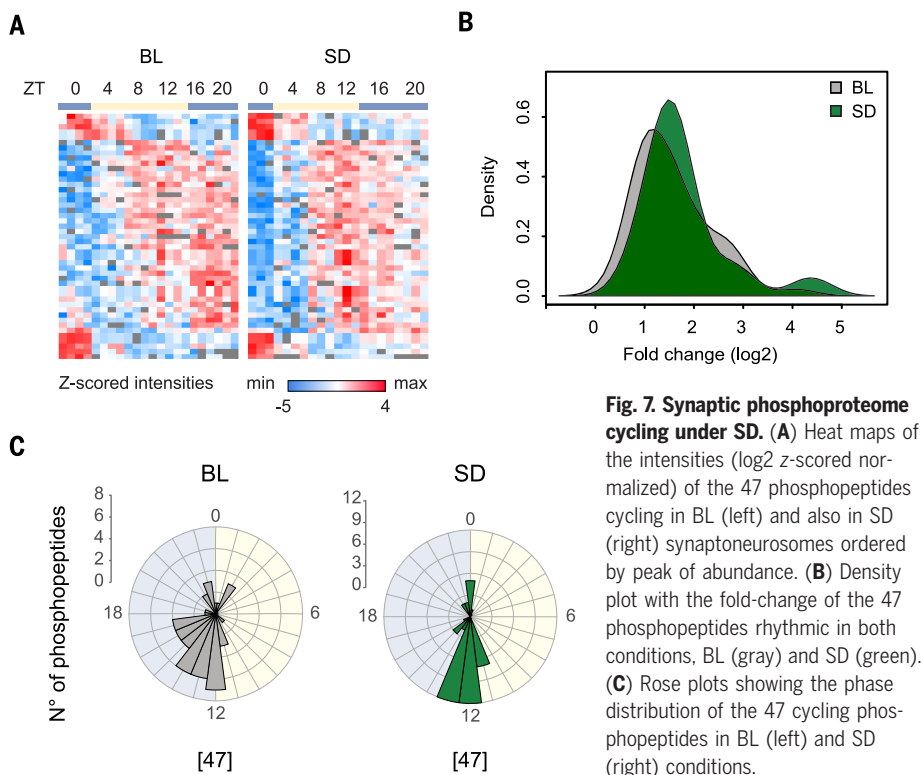
Isolated synaptoneurosome and total forebrain samples were lysed [0.1 M Tris-HCl

(pH 7.6) and 4% SDS], sonicated in a bioruptor (4°C for 15 min or until homogenous suspension was formed) and boiled at 95°C for 5 min. A total of 1 mg protein lysate was treated first with 1 µl DTT (1 M) followed by 10 µl 2-Chloroacetamide (0.5 M). Each treatment was performed at room temperature (RT; 22°C) for 20 min. The lysates were precipitated with acetone and phosphopeptides were enriched using the EasyPhos method as described (3). In detail, pellets were resuspended in 500 µl trifluoroethanol (TFE) buffer prior to the addition of digestion enzymes (trypsin and LysC) 1:100 (protein:enzyme). After overnight incubation at 37°C with rapid agitation (1500 rpm) 150 µl 3.2 M KCl, 55 µl of 150 mM KH_2PO_4 , 800 µl 100% acetonitrile (ACN), and 95 µl 100% trifluoroacetic acid (TFA) were added to the peptides and incubated at RT for 5 min at 1600 rpm prior to centrifugation. The peptide supernatant was transferred to a clean 2 ml tube, TiO_2 beads subsequently added at a ratio of 10:1 beads/protein, and incubated at 40°C for 5 min at 2000 rpm. Beads with bound phosphopeptides were pelleted by centrifugation for 1 min at 3500 g and the supernatant was discarded. Beads were then resuspended in wash buffer (60% ACN and 1% TFA) and transferred to a clean 2 ml tube, and washed further four times with 1 ml of washing buffer. After the last wash, beads were resuspended in transfer buffer (80% ACN and 0.5% acetic acid) and transferred on top of C8 (3M Empore) StageTips. Phosphopeptides were eluted with 60 µl elution buffer [40% ACN and 15% NH_4OH (25%, HPLC grade)] and collected in clean PCR

tubes, concentrated in a SpeedVac for 15 min at 45°C, and acidified with 10 µl of 10% TFA. Peptides were then desalted using StageTips with two layers of styrenedivinylbenzene-reversed phase sulfonated (SDB-RPS; 3M Empore), washed twice with wash buffer (0.2% TFA) and once with isopropanol containing 1% TFA. Peptides were eluted by adding 60 µl SDB-RPS elution buffer [80% ACN, 1.25% NH_4OH (25%, HPLC grade)] and immediately concentrated in a SpeedVac for 30 min at 45°C. Concentrated peptides were then resuspended in a buffer containing 2% ACN and 0.1% TFA prior to LC-MS/MS analysis.

LC-MS/MS analysis and data processing

Phosphopeptides were loaded onto a 50 cm reversed-phase column (diameter 75 µm; packed in-house with 1.9 µm C18 ReproSil particles [Dr. Maisch GmbH]). The temperature of the column oven was maintained at 60°C. The column was mounted to an EASY-nLC 1200 system (Thermo Fisher Scientific). The peptides were eluted with a binary buffer system consisting of buffer A (0.1% formic acid) and buffer B (80% ACN and 0.1% formic acid). A gradient length of 140 min was chosen (5 to 65% buffer B for 130 min followed by 10 min 80% buffer B) with a flow rate of 300 nl/min. Peptides were analyzed in a Q Exactive HF (synaptoneurosomes) and Q Exactive HF-X (total forebrain) mass spectrometer (MS) (Thermo Fisher Scientific) coupled to the nLC. Full scans [300 to 1600 mass/charge ratio (m/z), $R = 60,000$ at 200 m/z] were obtained at a target of 3e6 ions. The 10 most abundant ions were selected and fragmented with higher-energy collisional dissociation (HCD) (target 1e5 ions, maximum injection time 120 ms, isolation window 1.6 m/z , normalized collision energy 25% underfill ratio 40%) followed by the detection in the Orbitrap ($R = 15,000$ at 200 m/z). Raw MS data files were processed using MaxQuant [version 1.5.5.6 and 1.5.5.12 (43)] with the Andromeda search engine using false discovery rate (FDR) < 0.01 at protein, peptide, and modification level. The default settings were used with the following modifications: (i) the variable modification methionine (M), acetylation (protein N-term), as well as phosphorylation (STY) and the fixed modification carbamidomethyl (C) were selected, (ii) only peptides with a minimal length of seven amino acids were considered, and (iii) the “match between run” option was enabled with a matching time window of 0.7 min. For protein and peptide identification we used the UniProt database from mouse (September 2014) containing 51,210 entries. Each raw file was treated as one experiment. The following samples were not considered for the final analysis due to low identification number and outlier clustering within the replicates: replicate 4 of ZT16, 3 of ZT20 of the base line group and



replicate 1 of ZT0, 4 of ZT8, and 2 of ZT12 of the sleep deprived group.

Bioinformatic and statistical analyses

Processed data was uploaded in the Perseus software (14) to perform bioinformatical analyses. First, reverse sequences and potential contaminants were removed from the total matrix. Then phosphopeptides intensities were log2 transformed and after expanding the site table, phosphopeptide entries without intensities in <12 samples independently in each data set (SD/BL) were removed. Following this, a normalization was applied to each sample individually: the intensity of every phosphopeptide was divided by the median intensity of all phosphopeptides in the same sample. Relative enrichment of UniProt KB -Keywords annotations in the total BL phosphoproteome dataset was achieved using Fisher's exact test enrichment analyses (FDR < 0.02) comparing to a total in silico mouse gene list generated by Perseus, both datasets were matched using gene names. Cycling analyses were performed as described using the computational platform Perseus (2, 14). Phosphopeptides with a $q < 0.05$ were classified as "cycling." Hierarchical clustering was performed in a phase preserving manner by which the order of elements is restricted to that determined by the output of the periodicity analysis. The proteome and the phosphoproteome data were matched using Uniprot ID. Amplitudes were calculated as the difference between maximum and minimum values across all the time points using the mean

of log2 intensities for the biological replicates. Cycling phosphopeptides were divided in two groups depending on their peak of phosphorylation ("phase"): one group containing phosphopeptides which peak between ZT18 to ZT6 and the other group comprising phosphopeptides which peak between ZT6 to ZT18. Relative enrichment of UniProt KB -Keywords annotations for both groups was individually performed using Fisher's exact test enrichment analyses ($P < 0.05$) comparing the protein annotations of both groups to the protein annotations of the total cycling phosphopeptides.

Phosphopeptides belonging to kinases and phosphatases (using Keyword and manual annotations) were extracted from the total list of phosphopeptides and collapsed to protein entries (kinases). Kinases and phosphatases with at least one cycling phosphopeptide (cycling analysis, $q < 0.05$) were defined as "cycling." Gene name lists of all quantified kinases and of cycling kinases were submitted to www.kinhub.org to annotate kinases to the major families and to generate the kinase trees. Percentages of total quantified and cycling kinases in each family were calculated based on the entries for each family designated in www.kinhub.org.

Integrated analysis and visualization of phosphoproteomic data and protein-protein interaction network

Interactions for mouse were downloaded from STRING (v10.5) and filtered for high-confidence edges with scores of at least 0.9. Since the

node identifiers in the network are Ensemble protein IDs (ENSP), the protein groups in the phosphoproteomics data were mapped ENSP IDs using the UniProt annotations in Perseus. After annotating the nodes of the network with the data, signaling functionality scores were calculated using PHOTON. In brief, for each protein in the network a moderated t test was performed on the phosphorylation found on its neighbors in the network. Using a permutation-based FDR approach the resulting P value was corrected for multiple testing and transformed into a final score. Intuitively, proteins with high/low scores promote the phosphorylation of their neighbors. Cycling analysis was performed on the PHOTON scores to identify kinases with an oscillatory signaling functionality score ($q < 0.05$, period 24 hours). For the visualization, all cycling kinases and their interactions were extracted from the network. The subnetwork was overlaid with phosphorylation data from cycling sites and plotted using the d3.js library and manual layouting.

For the KSEA prediction, 1,664 site-specific mouse kinase substrate interactions were downloaded from phosphosite.org (44). The 7257 phosphopeptides could be mapped to 84 distinct kinase-substrate interactions. KSEA z-scores were calculated for all kinases in the network (17, 18), resulting in 37 kinase activity profiles with at least 3 valid values. After z-scoring the profiles, periodicity analysis was performed and cycling ($q < 0.05$, period 24 hours) kinase activity profiles were visualized in a scatter plot.

Western blotting and antibodies

Synaptoneurosome lysates were loaded onto 4 to 12% Bis-Tris NuPAGE gels (Thermo Fisher Scientific). After electrophoresis proteins were transferred to a nitrocellulose membrane and blocked in 5% BSA in TBS with 0.1% Tween-20 for 1 hour at room temperature. The incubation with the primary antibody was done overnight at 4°C following supplier instructions. Antibodies were obtained from Cell Signaling: CAMKII pT286 (12716), GSK3β pS9 (5558), and PKC pS660 (9371). The same secondary antibody against rabbit was used for all phospho-specific antibodies (GE Healthcare, NA934V) and was incubated for 1 hour at RT. Loading control was done with an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) conjugated with horseradish peroxidase from ThermoFisher Scientific (MA5-15738-HRP). Densitometric quantification was performed using the ImageJ software.

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SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/366/6462/eaav3617/suppl/DC1
Figures S1 to S13
Tables S1 to S6

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RESEARCH ARTICLE SUMMARY

IMMUNOLOGY

Migratory DCs activate TGF- β to precondition naïve CD8⁺ T cells for tissue-resident memory fate

Vinidhra Mani, Shannon K. Bromley, Tarmo Äijö, Rut Mora-Buch, Esteban Carrizosa, Ross D. Warner, Moustafa Hamze, Debattama R. Sen, Alexandra Y. Chasse, Alina Lorant, Jason W. Griffith, Rod A. Rahimi, Craig P. McEntee, Kate L. Jeffrey, Francesco Marangoni, Mark A. Travis, Adam Lacy-Hulbert, Andrew D. Luster, Thorsten R. Mempel*

INTRODUCTION: Successful immune responses to infections generate memory T cells that provide enhanced protection from reinfection by the same pathogen. Some memory cells continually recirculate through tissues via the blood and lymph, whereas others establish local residence. This second population includes CD8⁺ tissue-resident memory T cells that seed the epithelial layers of barrier tissues (eT_{RM} cells), such as the skin. These cells constitute a highly sensitive sentinel system, which responds to reencounters with cognate pathogen-derived antigens by triggering a local inflammatory response to rapidly contain the infection. The formation of eT_{RM} cells requires transforming growth factor β (TGF- β), a broadly expressed cytokine with a wide range of functions in the immune system. Secreted TGF- β is abundant in tissues, but its biological activity is tightly regulated

by release of the active cytokine from the latent pro-complex. Binding to α_V -integrins facilitates TGF- β release for activity in the immune system. However, the cellular mechanisms of its release, as well as the sites of CD8⁺ T cell exposure driving the formation of eT_{RM} cells, are unknown.

RATIONALE: Enhancing eT_{RM} cell formation is desirable in the context of vaccines designed to protect against pathogens that infect by way of barrier tissues. Conversely, attenuating eT_{RM} cell formation may be therapeutic for diseases, such as psoriasis, in which eT_{RM} cells play pathogenic roles. Understanding how TGF- β is activated and where it acts on T cells to enable eT_{RM} differentiation may inform new approaches to selectively amplify or disrupt this process without the predicted systemic effects of globally perturbing TGF- β activity. We hy-

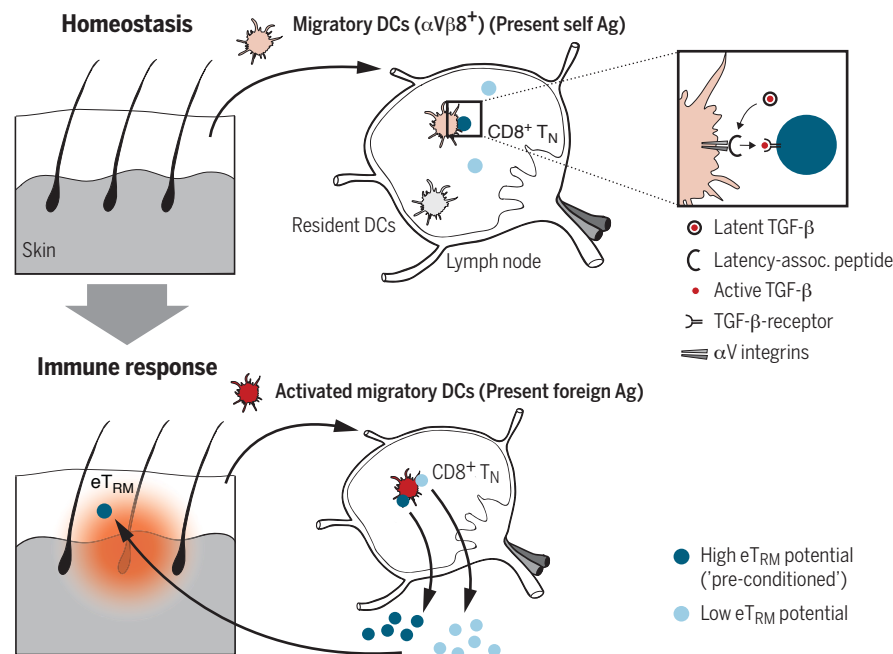
pothesized that α_V -integrin-expressing dendritic cells (DCs) activate and present TGF- β to CD8⁺ T cells to enable eT_{RM} cell formation.

RESULTS: Upon deleting α_V integrins from CD11c⁺ DCs in mice, we observed a pronounced reduction in the number of CD8⁺ T cells in the epidermis, whereas the numbers of dermal CD8⁺ T cells and other skin immune cells were unchanged. The same selective defect was apparent after different forms of skin immune challenge, including DNA vaccination,

indicating that the de novo formation of eT_{RM} cells was disrupted. Unexpectedly, neither expression of α_V integrins on DCs in skin-draining lymph nodes during priming of T cell responses, nor on DCs in the skin, was required for generation of eT_{RM} cells. Instead, the exposure of resting naïve CD8⁺ T cells to α_V -expressing DCs during immune homeostasis preconditioned them for effective formation of eT_{RM} cells upon activation. An examination of the genomic accessibility of naïve cells suggested that TGF- β signals enabled by α_V -expressing DCs prime genes involved in eT_{RM} cell formation for their more rapid induction. This reversible preconditioning effect was mediated by migratory DCs and occurred in lymph nodes, but not in spleen, as both exposure of naïve T cells to TGF- β and the formation of eT_{RM} cells in the skin were strongly impaired in the absence of lymph nodes or of CCR7-dependent DC migration from skin to lymph nodes. eT_{RM} formation was also reduced when expression of major histocompatibility complex class I (MHC I) molecules and α_V integrins was segregated on individual DCs, indicating that exposure of naïve CD8⁺ T cells to TGF- β occurs in the context of noncognate, yet MHC I-dependent, physical interactions with migratory DCs.

CONCLUSION: Naïve CD8⁺ T cells are preconditioned for the formation of skin eT_{RM} cells by DCs that migrate from nonlymphoid tissues to lymph nodes at steady state. These DCs both activate and present TGF- β to naïve T cells, exemplifying how this cytokine's potent biological activities can be limited to specific contexts through its requirement for extracellular processing. In this way, individual T cells appear to be actively preconditioned for a specific differentiation path already at the naïve cell stage. This is in contrast to the general expectation that the preimmune T cell repertoire is uniform in its potential to differentiate into various effector and memory cell subsets upon activation. ■

The list of author affiliations is available in the full article online.
*Corresponding author. Email: tmempel@mgh.harvard.edu
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Migratory DCs activate TGF- β in lymph nodes to precondition naïve CD8⁺ T cells for eT_{RM} cell formation. During homeostasis, migratory DCs that express α_V integrins, including $\alpha_V\beta_8$, but not resident DCs, activate TGF- β and present it to naïve CD8⁺ T cells (T_N) during noncognate interactions. Preconditioned naïve T cells then effectively become epithelial resident memory T (eT_{RM}) cells upon immune challenge.

RESEARCH ARTICLE

IMMUNOLOGY

Migratory DCs activate TGF- β to precondition naïve CD8⁺ T cells for tissue-resident memory fate

Vinidhra Mani^{1,2}, Shannon K. Bromley^{1,3}, Tarmo Äijö⁴, Rut Mora-Buch^{1,3}, Esteban Carrizosa^{1,3,5}, Ross D. Warner¹, Moustafa Hamze^{1,3}, Debattama R. Sen^{2,6}, Alexandra Y. Chasse¹, Alina Lorant⁷, Jason W. Griffith^{1,3}, Rod A. Rahimi^{1,3}, Craig P. McEntee⁸, Kate L. Jeffrey^{3,9}, Francesco Marangoni^{1,3,*}, Mark A. Travis⁸, Adam Lacy-Hulbert⁷, Andrew D. Luster^{1,3}, Thorsten R. Mempel^{1,3,†}

Epithelial resident memory T (eT_{RM}) cells serve as sentinels in barrier tissues to guard against previously encountered pathogens. How eT_{RM} cells are generated has important implications for efforts to elicit their formation through vaccination or prevent it in autoimmune disease. Here, we show that during immune homeostasis, the cytokine transforming growth factor β (TGF- β) epigenetically conditions resting naïve CD8⁺ T cells and prepares them for the formation of eT_{RM} cells in a mouse model of skin vaccination. Naïve T cell conditioning occurs in lymph nodes (LNs), but not in the spleen, through major histocompatibility complex class I-dependent interactions with peripheral tissue-derived migratory dendritic cells (DCs) and depends on DC expression of TGF- β -activating α_V integrins. Thus, the preimmune T cell repertoire is actively conditioned for a specialized memory differentiation fate through signals restricted to LNs.

T cell immune responses contract after successful control of an infection, but various types of memory cells persist and provide enhanced protection from reencounters with the respective pathogen. Some memory cells continually recirculate through lymphoid and nonlymphoid tissues via the blood and the lymph, whereas so-called tissue-resident memory T (T_{RM}) cells adopt states of more permanent local residence (1). This latter population includes CD8⁺ cells that coexpress the tissue residency markers CD69 and CD103 (α E integrin) and populate the epithelial layers of environmental barrier tissues, such as the skin (2, 3). These epithelial T_{RM} (eT_{RM}) cells form a highly sensitive sentinel system and respond to reencounter with their cognate pathogen-derived antigen with direct antiviral or antimicrobial effector activities. Additionally, eT_{RM} cells

trigger local inflammatory responses that efficiently recruit circulating memory and other immune cells to rapidly contain the infection (4–6). eT_{RM} cells are thought to develop locally at their site of residence from uncommitted memory precursors, which acquire the ability to respond to transforming growth factor β (TGF- β) through coordinated downregulation of the T-box factors T-bet and eomesodermin (Eomes). TGF- β , in turn, induces the expression of *Cd103* and other tissue residency-associated genes and enables long-term persistence of eT_{RM} cells in the epithelium (7–11).

TGF- β is a pleiotropic cytokine with a broad range of functions in the immune system. It is widely expressed and secreted in its latent form. As such, it is abundant in most tissues where it is bound to cell surfaces and extracellular matrix by “milieu” factors such as glycoprotein-A repetitions predominant protein (GARP) or latent TGF- β -binding proteins (LTBPs), respectively. The cytokine acquires its biological activity only upon simultaneous binding by integrins, which allows for the generation of force to distort the TGF- β prodomain. This, in turn, triggers the release of the growth factor domain that binds to TGF- β receptors (12). TGF- β activity in the immune system is enabled by α_V integrins expressed both by hematopoietic and nonhematopoietic cells (13). Keratinocyte-expressed $\alpha_V\beta_6$ and $\alpha_V\beta_8$ integrins, for instance, activate the pool of TGF- β that maintains the stable, long-term residence of Langerhans cells and eT_{RM} cells in skin (14). However, the relevant micro-anatomical sites of CD8⁺ T cell exposure to

TGF- β , as well as the cellular mechanisms underlying its activation, which serve to initiate and drive eT_{RM} cell differentiation during the formation of T cell memory, remain unknown.

Efficient eT_{RM} cell formation in skin requires dendritic cell expression of α_V integrins

To test whether α_V -expressing dendritic cells (DCs) activate TGF- β to facilitate eT_{RM} cell differentiation, we crossed mice with *loxP*-flanked *Itgav* alleles (15) to *Cd11c*^{Cre} bacterial artificial chromosome (BAC)-transgenic mice, in which Cre recombinase is active in >95% of all conventional DCs (16). In the resulting *Cd11c*^{Cre} $\times$ *Itgav*^{f/f} mice (hereafter referred to as “ α_V -ADC” mice), α_V protein was absent from the majority of DCs (fig. S1, A and B). The deletion of α_V did not disrupt DC homeostasis, because the proportion of various DC populations in skin and skin-draining LNs was unchanged compared to *Cd11c*^{Cre} $\times$ *Itgav*^{+/+} littermate control (“WT”) mice (fig. S1, C and D). Mice whose DCs lack the β_8 integrin that pairs with α_V to form the primary TGF- β -activating $\alpha_V\beta_8$ heterodimer expressed in immune cells show signs of immune activation, possibly resulting from the impaired formation of peripheral regulatory T (T_{reg}) and T helper 17 (T_H17) cells in the intestine (17, 18). Similarly, young α_V -ADC mice showed moderate hypercellularity, expansion of CD44^{hi} CD62L^{lo} CD8⁺ T cells, and enhanced cytokine expression by CD4⁺ cells in spleen, but not in LNs. There was also an increase in serum immunoglobulin E (IgE) and IgG in these mice (fig. S1, E to J). However, no histological signs of inflammation were observed in the colon and skin (fig. S1K), and mice displayed no signs of disease, such as weight loss, until at least 6 months of age.

Notably, already in young α_V -ADC mice, histological examination revealed a pronounced and selective loss of CD8⁺ T cells in the epidermis, but not in the dermis, whereas the density of CD3^{bright} CD8⁺ dendritic epidermal T cells (DETCs) in the skin epithelium was unchanged (Fig. 1, A to C). The frequency of CD4⁺ T cells, which are only found within the dermis in mice (19), was also unchanged (fig. S2A). Accordingly, there was a selective decrease in the frequency of skin CD8⁺ T cells coexpressing CD69 and CD103/ α E integrin, indicative of epidermal residence. By contrast, CD4⁺ and $\gamma\delta$ T cells coexpressing CD69 and CD103, as well as DCs expressing CD103, were present at frequencies similar to those in control mice (Fig. 1, D and E, and fig. S2B). An even more pronounced defect in eT_{RM} cell differentiation was evident following skin inflammation induced by either mechanical skin irritation using a tattooing device (20) or treatment with the contact sensitizer dinitrofluorobenzene (DNFB) (Fig. 1, F to J, and fig.

¹Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital, Boston, MA, USA.

²Immunology Graduate Program, Harvard Medical School, Boston, MA, USA. ³Harvard Medical School, Boston, MA, USA. ⁴Center for Computational Biology, Flatiron Institute, New York, NY, USA. ⁵Bluebird Bio, 60 Binney Street, Cambridge, MA 02142, USA.

⁶Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA, USA. ⁷Benaroya Research Institute, Seattle, WA, USA. ⁸Lydia Becker Institute of Immunology and Inflammation, Wellcome Trust Centre for Cell-Matrix Research, Faculty of Biology, Medicine and Health Manchester Academic Health Science Centre, University of Manchester, Manchester, UK.

⁹Gastrointestinal Unit and Center for the Study of Inflammatory Bowel Disease, Massachusetts General Hospital, Boston, MA, USA.

*Present Address: University of California Irvine, Irvine, CA 92697, USA.

†Corresponding author. Email: tmempel@mgh.harvard.edu

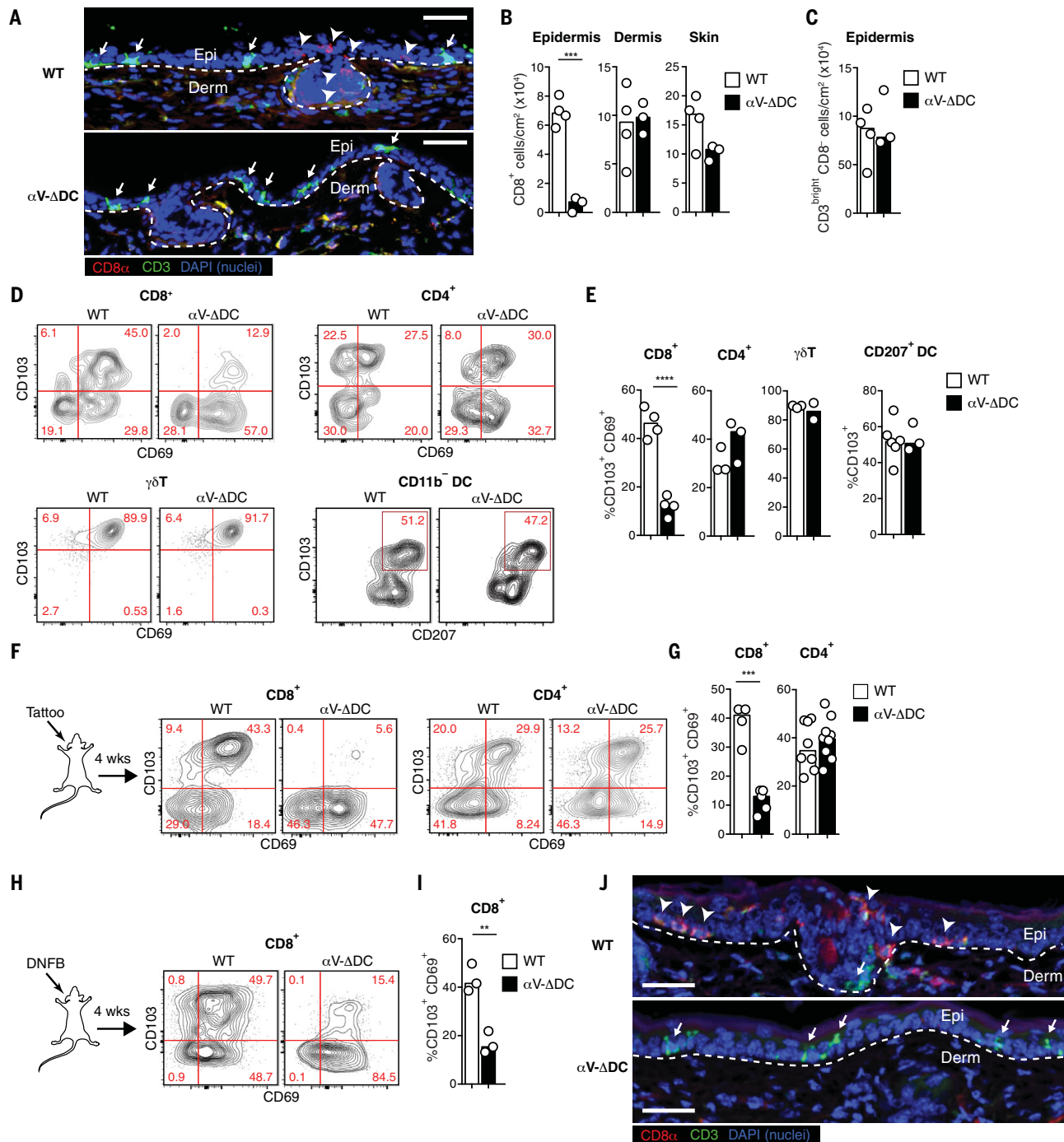


Fig. 1. Defective epidermal T_{RM} cell formation in the absence of αV integrins on DCs. (A) Histological cross-sections from ear skin of 8- to 10-week-old $\alpha V\text{-}\Delta DC$ and WT littermate control mice. Arrowheads indicate $CD8^+ CD3^{\text{dim}}$ T_{RM} cells in the epidermis of WT, but not $\alpha V\text{-}\Delta DC$ mice, whereas arrows indicate $CD3^{\text{bright}}$ DETCs present in both strains. Dashed lines indicate the dermal-epidermal border. Scale bar, 50 μm . Epi: epithelium; Derm: dermis. (B and C) Density of $CD8^+$ T cells in epidermis, dermis, and total skin (B) and of $CD3^{\text{bright}}$ DETCs in epidermis (C). Data are medians and replicates and are representative of two independent experiments. Each symbol represents the mean of three to four values per animal obtained from different histological sections. (D and E) Expression of the tissue residence marker CD103 (as well as CD69 on T cells) on the indicated immune cell subsets (Thy1 $^+$ $CD8\beta^+$ T cells, Thy1 $^+$ $CD4^+$ T cells, Thy1 $^+$ TCR δ^+ $\gamma\delta T$ cells,

$CD11c^+$ MHC II $^+$ $CD11b^-$ DCs) from skin of $\alpha V\text{-}\Delta DC$ and WT littermate control mice. Data are medians and replicates and are representative of two independent experiments. (F to I) Expression of CD69 and CD103 on skin $CD8^+$ or $CD4^+$ T cells 4 weeks after sterile inflammation induced by mechanical irritation with a tattooing device [(F) and (G)] or topical DNFB treatment [(H) and (I)] in 8- to 10-week-old mice. Data are medians and replicates and are representative of five [(F) and (G)] or three [(H) and (I)] independent experiments. (J) Ear skin 4 weeks after DNFB treatment. Note the enrichment of $CD8\beta^+ CD3^{\text{dim}}$ eT_{RM} cells (arrowheads) in WT mice, but their absence in the epithelium of $\alpha V\text{-}\Delta DC$, which is instead still populated by $CD3^{\text{bright}}$ DETC (arrows). Data are representative of two animals per group. Scale bar, 50 μm . ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ [two-tailed unpaired Student's t tests in (B), (C), (E), (G), and (I)].

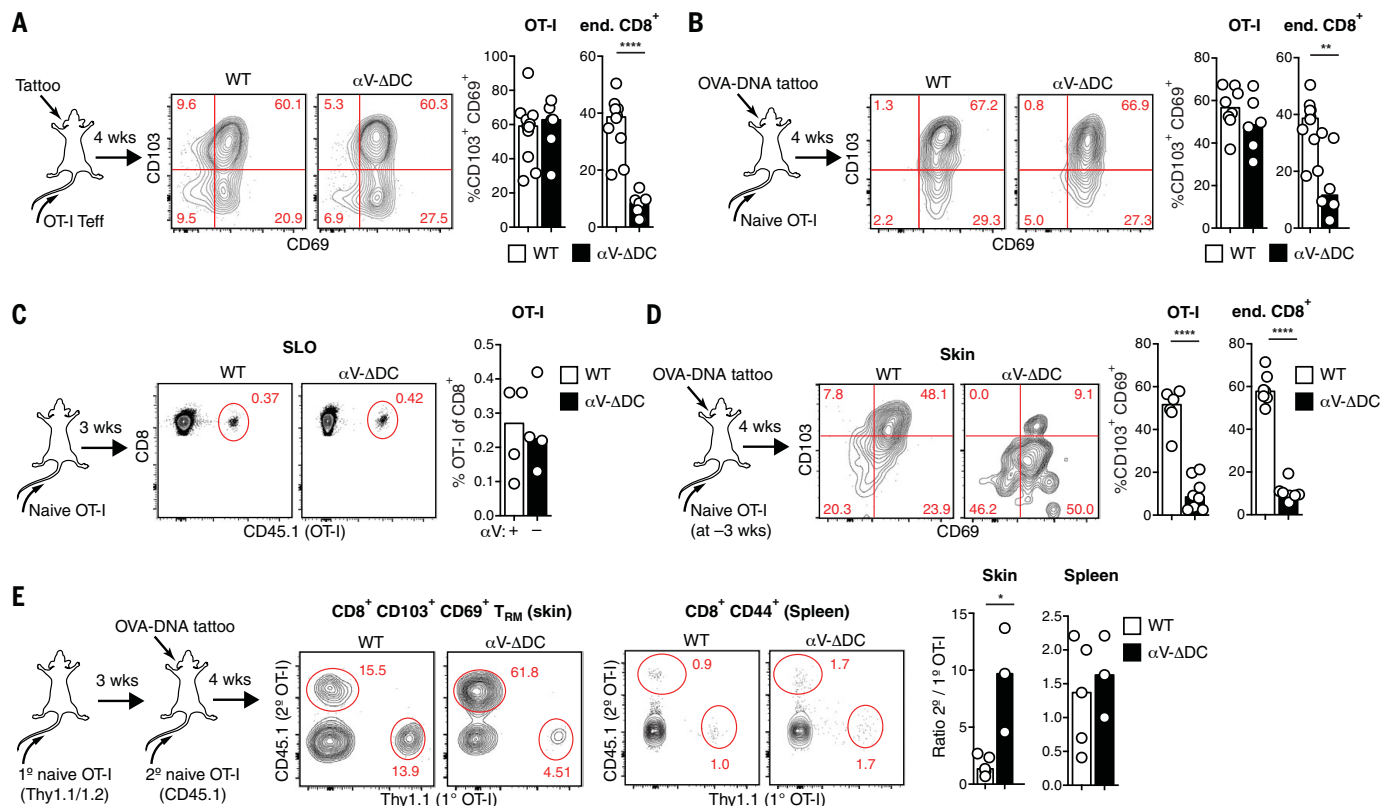


Fig. 2. DC-expressed α_V integrins condition naïve CD8⁺ T cells for epithelial T_{RM} cell formation prior to vaccine-induced activation.

(A) 10^6 Thy1.1/2 congenic ex vivo activated OT-I effector cells were intravenously injected into Thy1.2 $\alpha V\text{-}\Delta DC$ or WT recipients, whose ears were simultaneously inflamed through tattoo injury. After 4 weeks, CD69 and CD103 expression was assessed on transferred OT-I and host CD8⁺ T cells in skin. Data are medians and replicates and are representative of six independent experiments. (B) 10^5 CD44^{lo} CD62L^{hi} naïve OT-I cells were adoptively transferred into $\alpha V\text{-}\Delta DC$ or WT recipients, whose ears were simultaneously tattooed with OVA-encoding plasmid DNA to prime OT-I in skin-draining LNs. After 4 weeks, CD69 and CD103 expression was assessed on transferred OT-I and host CD8⁺ T cells in skin. Data are medians and replicates and are representative of five independent experiments. (C and D) 10^6 CD44^{lo} CD62L^{hi} naïve OT-I cells were adoptively transferred into $\alpha V\text{-}\Delta DC$ or WT recipients. Three weeks later, OT-I cell

frequency in pooled LNs and spleen (SLO) was determined in some animals (C), and the remaining animals were vaccinated by ear tattoo with OVA-encoding plasmid DNA (D). After 4 weeks, CD69 and CD103 expression was assessed on transferred OT-I and host CD8⁺ T cells in skin. Data are medians and replicates and are representative of four independent experiments. (E) 10^6 Thy1.1/2 congenic CD44^{lo} CD62L^{hi} naïve OT-I cells (1° OT-I) were adoptively transferred into $\alpha V\text{-}\Delta DC$ or WT recipients. Three weeks later, a second batch of 10^5 CD45.1 congenic OT-I cells (2° OT-I) was transferred into the same recipients and mice were vaccinated by ear tattoo with OVA-encoding plasmid DNA. After 4 weeks, the ratios of OT-I derived from the second and the first injected batch were assessed in the pools of CD69⁺ CD103⁺ T_{RM} cells in skin and of CD44⁺ CD8⁺ T cells in spleen. Data are medians and replicates and representative of two independent experiments. * p < 0.05, ** p < 0.01, **** p < 0.0001 [two-tailed unpaired Student's t tests in (A) to (E)].

S2, C and D). Thus, the expression of TGF- β -activating α_V integrins on DCs is critical for the efficient formation of CD8⁺ e T_{RM} cells in the stratified epithelium, but not for other local CD103⁺ immune cells in skin.

Naïve T cells are preconditioned for e T_{RM} cell formation by DC prior to antigenic priming

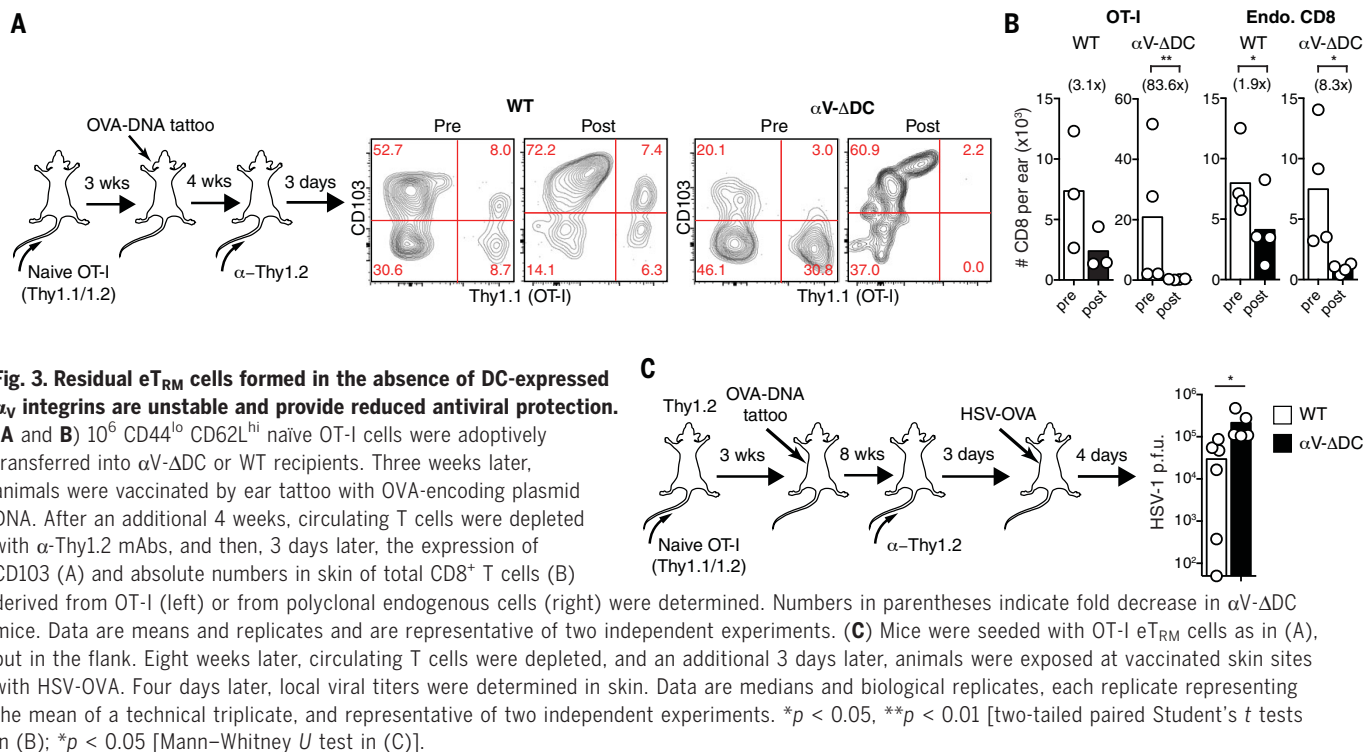
Terminal e T_{RM} cell differentiation is thought to be initiated locally in skin from uncommitted memory precursors (7, 11). We therefore hypothesized that dermal α_V -expressing DCs may enhance e T_{RM} cell formation by generating a subepithelial pool of active TGF- β to which CD8⁺ memory precursor T cells would then be exposed upon extravasation into the inflamed skin. However, when we intravenously injected

ex vivo-activated ovalbumin (OVA)-specific effector CD8⁺ T (T_{EFF}) cells from OT-I T cell receptor (TCR) transgenic mice, they differentiated into CD69⁺ CD103⁺ e T_{RM} cells in the inflamed skin of $\alpha V\text{-}\Delta DC$ and WT hosts with similar efficiencies (Fig. 2A and fig. S2E). Thus, the local activation of TGF- β by α_V -expressing DCs in skin is not required for e T_{RM} cell formation.

Alternatively, we hypothesized that CD8⁺ T cells may be exposed to TGF- β activated by DCs during antigenic priming in skin-draining LNs, which might endow them with enhanced e T_{RM} cell differentiation or skin-homing capacity [e.g., through the TGF- β -dependent induction of T cell ligands for selectins expressed on inflamed skin endothelium (21)]. However,

the transfer of naïve OT-I T cells and vaccination with OVA-expressing plasmid DNA also generated similar frequencies of e T_{RM} cells in the skin of $\alpha V\text{-}\Delta DC$ and WT hosts (Fig. 2B and fig. S2F).

Because TGF- β -activation by α_V -expressing DCs was important for e T_{RM} cell formation, but was not critical during T cell priming in draining LNs or during terminal differentiation at the effector site, we considered other contexts in which T cells may be exposed to TGF- β . Resting T cells depend on interactions with self-antigens presented by DCs for their maintenance (22). Thus, we hypothesized that α_V -expressing DCs may at the same time, during immune homeostasis, expose naïve CD8⁺ T cells in secondary lymphoid organs



(SLOs) to active TGF- β and thereby precondition them for enhanced eTRM cell formation upon eventual foreign antigen encounter. We therefore transferred naïve OT-I cells into α_v -ΔDC mice where such preconditioning might be lost, or into WT mice where it would be sustained. After 3 weeks, at which time OT-I cell frequency among naïve CD8⁺ T cells in SLOs was comparable in both strains (Fig. 2C), we vaccinated mice with OVA-DNA via the ear skin. Four weeks after this regimen, we observed a reduction in the frequency of CD103⁺ OT-I eTRM cells at vaccinated skin sites in α_v -ΔDC (but not in WT hosts), which matched the reduction in the frequency of eTRM cells derived from the hosts' endogenous polyclonal CD8⁺ T cell populations (Fig. 2D). To control for potentially variable T cell priming efficiency in individual animals, we also transferred two batches of naïve OT-I cells into either α_v -ΔDC or WT mice. The first and second batches were injected 3 weeks and immediately before OVA skin vaccination, respectively (Fig. 2E). Cells from both batches formed eTRM cells at nearly equal efficiencies in WT mice, suggesting that their capacity to form eTRM cells had not changed over the course of 3 weeks. By contrast, cells from the first batch were tenfold less efficient at forming eTRM cells than those from the second batch in α_v -ΔDC hosts. Thus, the capacity of naïve CD8⁺ T cells to form eTRM cells declines over time when DCs do not express α_v integrins. No such bias in favor of more recently trans-

ferred cells was observed among circulating memory T cells in spleen (Fig. 2E).

Naïve cell preconditioning is required for optimal eTRM cell-mediated antiviral protection

A hallmark of eTRM cells is their resistance to antibody-mediated depletion (10, 19, 23). Accordingly, when we injected T cell-depleting antibodies into WT or α_v -ΔDC animals seeded with skin memory cells derived from naïve OT-I cells transferred 3 weeks before skin vaccination, we observed a loss of CD103⁺ dermal T_{RM} cells and a corresponding enrichment of CD103⁺ eTRM cells in all animals, as expected (Fig. 3A). The total numbers of remaining endogenous and OT-I CD8⁺ T cells in skin were each only moderately (two- to threefold) reduced in WT hosts. By contrast, the decrease in endogenous CD8⁺ T cell numbers was much more pronounced (eightfold) and OT-I cells were essentially eliminated in α_v -ΔDC hosts (Fig. 3B). Thus, even the few eTRM cells that had formed from “deconditioned” naïve precursors in α_v -ΔDC hosts were less stably resident within the epithelium. Consequently, eTRM cells derived from deconditioned precursors were less effective at protecting animals from challenge with OVA-expressing herpes simplex virus 1 (HSV-OVA) (Fig. 3C). Thus, continual exposure to DCs expressing TGF- β -activating α_v integrins sustained the capacity of naïve CD8⁺ T cells to efficiently form protective epidermal T_{RM} cells.

By contrast, the formation of circulating memory cells appeared to be unaffected.

α_v -expressing DCs regulate chromatin accessibility in preconditioned naïve CD8⁺ T cells

We hypothesized that exposure to active TGF- β may induce a permissive epigenetic state in resting CD8⁺ T cells that enhances their capacity to form eTRM cells upon activation. For instance, TGF- β may prepare genes relevant to eTRM cell formation for more rapid expression. We therefore purified CD44^{lo} naïve CD8⁺ T cells from young WT and α_v -ΔDC mice and investigated their genome-wide chromatin accessibility through the assay for transposase accessible chromatin using high-throughput sequencing (ATAC-seq). Among the 13 Mb of detected accessible chromatin (<0.04% of the mouse genome), 6.8% was differentially accessible, with 496 kb (3.8%) being more accessible in naïve CD8⁺ T cells from WT and 391 kb (3.0%) being more accessible in cells from α_v -ΔDC mice. Peak calling and merging yielded low numbers of differentially accessible regions (DARs) of DNA both in cells from WT and from α_v -ΔDC hosts (Fig. 4A and table S1). DARs in WT cells clustered around gene transcription start sites (TSSs), indicating that these were potentially expressed or ready to be expressed. By contrast, DARs in α_v -ΔDC cells were more frequently located distal to the TSS of the most proximal gene (Fig. 4B). These latter DARs were enriched in binding motifs

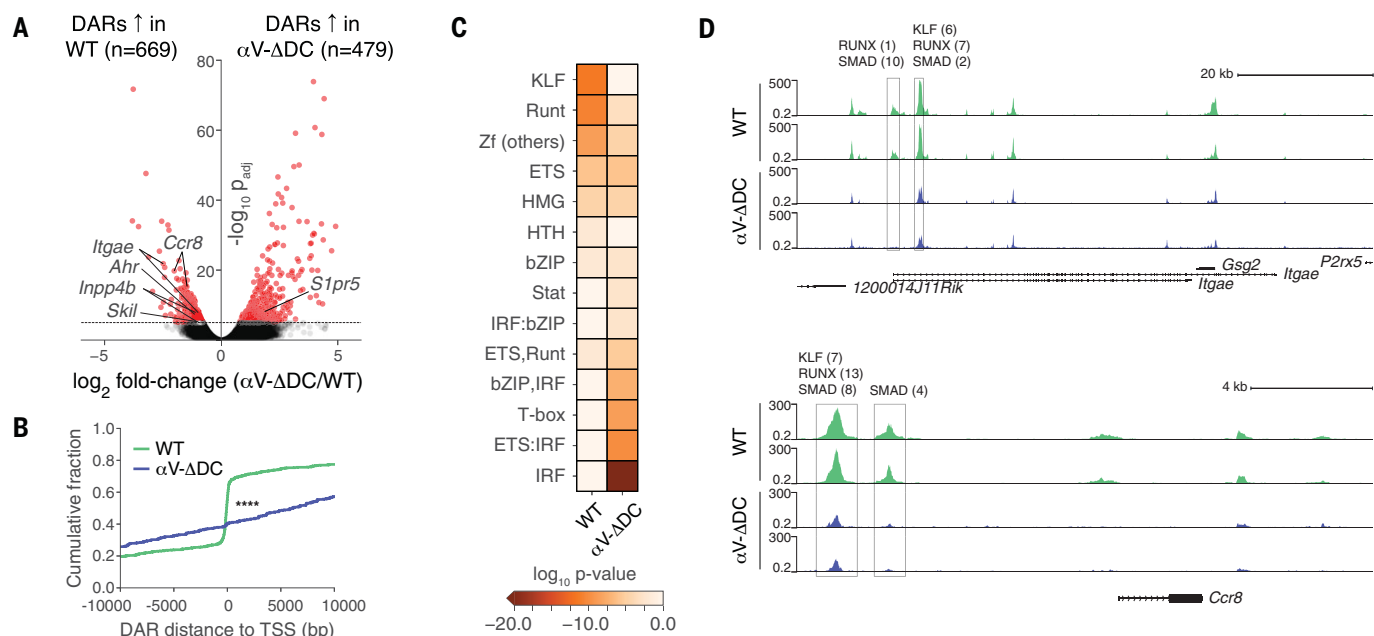


Fig. 4. Resting naïve CD8⁺ T cells are epigenetically conditioned by TGF-β signals in secondary lymphoid tissues. (A) Volcano plot of chromatin accessibility changes between CD44^{lo} naïve CD8⁺ T cells from WT and αV-ADC mice, considering merged DARs. Genes proximal to some selected DARs are indicated. (B) Cumulative distributions of distances from DARs in cells from WT (green) and αV-ADC (blue) animals to the closest transcription start site (TSS).

The two-sample Kolmogorov–Smirnov was used to compare cumulative distributions. (C) Enrichment of the indicated transcription factor binding motif families in DARs. (D) Normalized chromatin accessibility near the *Itgae* (top) and *Ccr8* (bottom) loci. The rectangles mark detected DARs. All analyses were performed on two mice per group with similar results. **** $p < 0.0001$ [two-sample Kolmogorov–Smirnov test in (B)].

for transcription factors (TFs) associated with cellular activation, such as interferon regulatory factors (IRFs) and T-box factors. By contrast, DARs in T cells from WT mice were enriched in binding motifs for the Krüppel-like family of TFs (KLFs) and other zinc finger (Zf) DNA-binding proteins, as well as for Runx family TFs, including Runx3 (Fig. 4C and fig. S3). Runx3 has recently been identified as an important positive regulator of eT_{RM} cell differentiation (11). Of note, Runx TFs interact with and enable the functions of TGF-β-regulated Smad TFs (24). Thus, the role of Runx3 during eT_{RM} cell differentiation may, at least in part, be based on its requirement for TGF-β-mediated gene regulation.

Enhanced accessibility of eT_{RM} cell genes in preconditioned naïve CD8⁺ T cells

The presence of Runx motifs in regions accessible in naïve cells from WT but not αV-ADC mice also suggests that some epigenetic changes driving eT_{RM} cell differentiation may already occur in resting T cells prior to activation. Indeed, among a set of 21 signature genes up-regulated during eT_{RM} cell differentiation in skin (7, 25), five were proximal to DARs in WT CD8⁺ T cells, including *Itgae* (encoding CD103), *Ahr*, and *Ccr8* (Fig. 4A). By contrast, the tissue egress-promoting gene *S1pr5*, which is down-regulated in eT_{RM} cells (7, 25), contained a DAR

upstream of its TSS in cells from αV-ADC mice (fig. S4A). DARs proximal to eT_{RM} cell genes mostly contained multiple clustered Runx, KLF, and Smad motifs (Fig. 4D and fig. S4A). Because KLFs also interact with Smad TFs and mediate TGF-β signaling (26), this pattern of motif enrichment suggested the involvement of TGF-β in the epigenetic changes that we observed in naïve CD8⁺ T cells. Accordingly, genes proximal to DARs in WT cells were most strongly associated with the TGF-β pathway and with cell cycle arrest among biological processes (fig. S4, B and C).

The preconditioned state is reversible and actively maintained in secondary lymphoid organs

From our analysis of publicly available gene expression data sets deposited through the Immunological Genome Project (www.immgen.org), and using the transcript levels of well-characterized naïve and effector cells genes as a reference, we found that some eT_{RM} cell genes proximal to DARs in WT cells were expressed in naïve CD8⁺ T cells (*Itgae*, *Skil*, *Inpp4b*), whereas others were not (*Ahr*, *Ccr8*) (Fig. 5A). Among the former, *Itgae* was highly expressed as integrin αE/CD103 protein on naïve CD8⁺, but not on CD4⁺ T cells in WT mice (Fig. 5B). Because CD103 expression by naïve CD8⁺ T cells has been shown to depend on TGF-β signals (27), we used it as a

surrogate marker of TGF-β-mediated conditioning. As predicted from our ATAC-seq analysis, CD103 expression was substantially reduced on naïve CD8⁺ T cells in αV-ADC mice, but not on CD8 single-positive (SP) thymocytes from the same animals (Fig. 5B). Because thymocyte expression of CD103 also depends on the cooperation of Runx3 and TGF-β signals (28), normal thymocyte expression may reflect alternative local mechanisms of TGF-β activation, such as α_V integrins expressed by thymic epithelial or other stromal cells.

The defect in CD103 expression on naïve CD8⁺ T cells from αV-ADC mice was not cell-intrinsic, because treatment with TGF-β in vitro induced their expression of CD103 at levels similar to those in WT T cells (fig. S5B). Also, CD103^{lo} cells from αV-ADC mice expressed CD103 upon adoptive transfer into WT hosts (Fig. 5C). Conversely, both polyclonal as well as clonal OT-I T cells from α_V-sufficient mice lost CD103 expression in αV-ADC hosts, indicating that expression must be actively maintained following induction (Fig. 5C-D). Notably, activated T cells transiently down-regulate TGF-β receptors (29). Accordingly, CD103 expression in CD8⁺ T cells was first lost upon antigenic priming in LNs before its eventual reexpression at considerably higher levels in the eT_{RM} cells that form in skin (fig. S5, C to E). Thus, following T cell

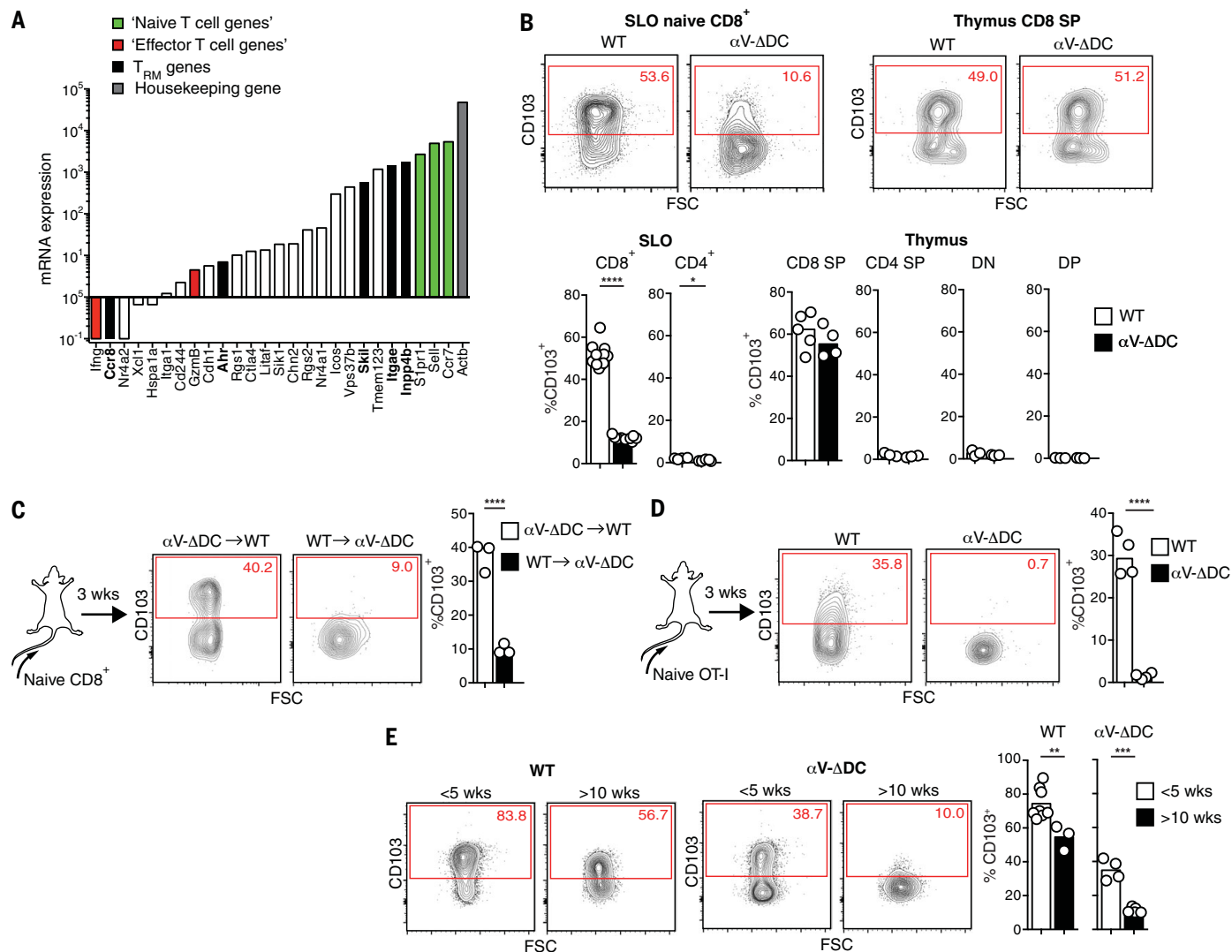


Fig. 5. CD103 is actively maintained on naïve CD8⁺ T cells through α_V -integrin-expressing DCs. (A) ImmGen database RNA-seq gene expression analysis of naïve CD8⁺ T cells. Green bars denote selected genes known to be expressed at the protein level in naïve T cells; red bars denote selected genes known to be expressed at very low levels or not to be expressed in naïve T cells. Black bars denote skin eT_{RM} cell genes having greater accessibility in naïve CD8⁺ T cells from WT as compared to α_V - Δ DC mice. White bars denote other skin eT_{RM} cell genes not differentially accessible in cells from WT compared to α_V - Δ DC mice. (B) Expression of CD103 on CD44^{lo} CD62L^{hi} naïve CD4⁺ and CD8⁺ T cells (pooled from LNs and spleen) and on thymocyte subsets from α_V - Δ DC and WT littermate control mice. Data are medians and replicates and are representative of four independent

experiments. (C) 10⁶ naïve polyclonal CD8⁺ T cells from α_V - Δ DC were adoptively transferred into CD45.1 congenic C57BL/6 mice or vice versa and reisolated 3 weeks later from pooled LNs and spleens for analysis of CD103 expression. Data are medians and replicates and are representative of three independent experiments. (D) 10⁶ naïve OT-I T cells were adoptively transferred into α_V - Δ DC or WT hosts and isolated from pooled LNs and spleens 3 weeks later for analysis of CD103 expression. Data are medians and replicates and are representative of three independent experiments. (E) CD103 expression on CD44^{lo} naïve CD8⁺ T cells from the peripheral blood of WT and α_V - Δ DC mice at less than 5 or more than 10 weeks of age. Data are medians and replicates. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 [two-tailed unpaired Student's *t* tests in (B) to (E)].

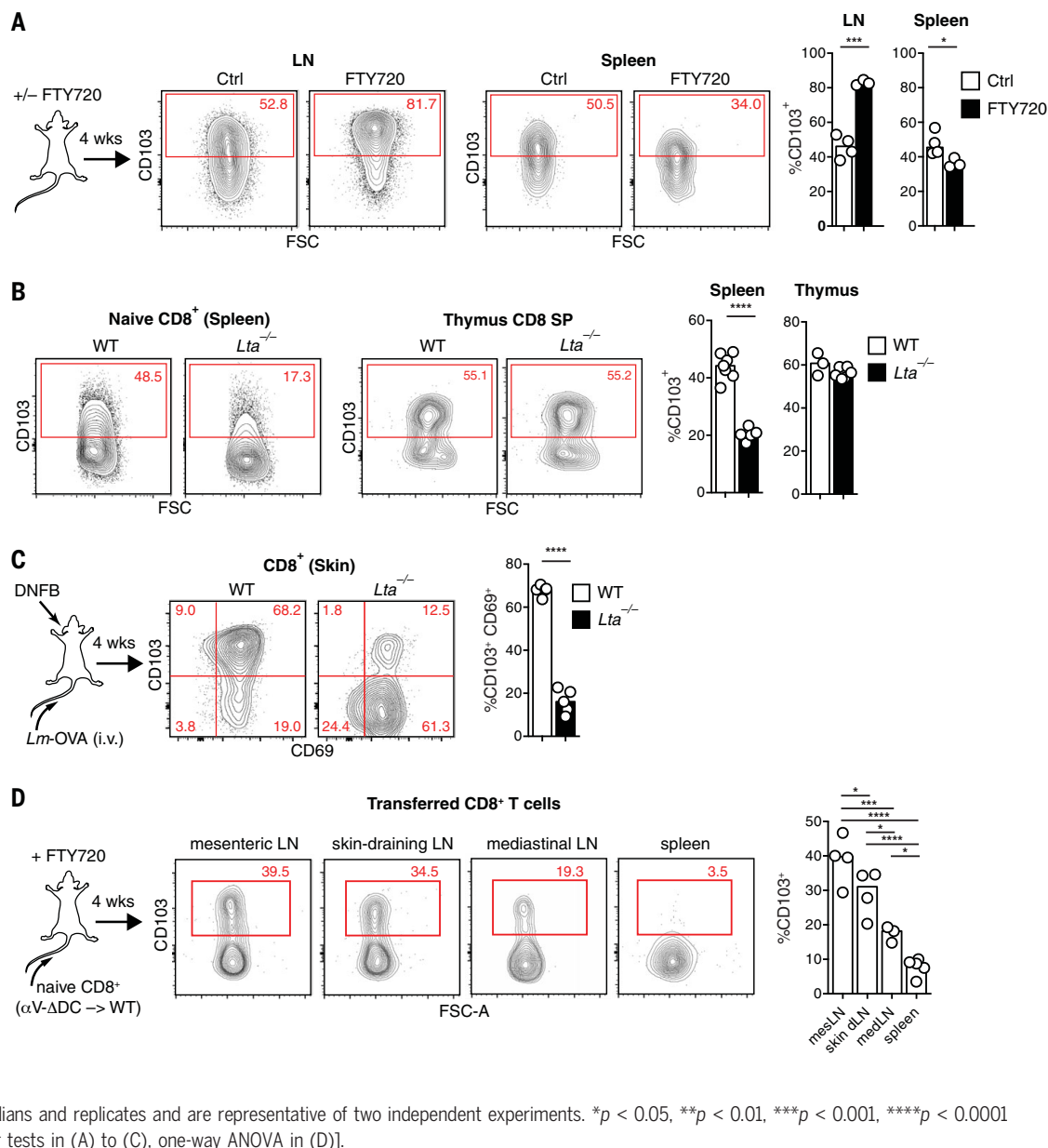
egress from thymus, DC-dependent exposure to active TGF- β in secondary lymphoid organs (SLOs) renders multiple genes important in their differentiation, including *Ilgae*, more accessible in naïve CD8⁺ T cells. Despite the transient loss of gene expression upon T cell activation, at least of *Cd103*, this enhanced accessibility potentially facilitates the accelerated transcriptional activation and more efficient formation of eT_{RM} cells.

Preconditioning occurs in lymph nodes, but not in the spleen

Because the majority of CD8 SP thymocytes expressed CD103, it was possible that some CD103⁺ naïve CD8⁺ T cells were recent thymic emigrants (RTEs) that transiently retained CD103 expression induced in the thymus independently of α_V -expressing DCs. Indeed, in WT mice younger than 5 weeks of age, in which the majority of peripheral T cells are

RTEs (30), CD103 expression on naïve CD8⁺ T cells was higher than in mice older than 10 weeks of age, when the thymic output rate has stabilized. This difference was even more pronounced in α_V - Δ DC mice, in which naïve CD8⁺ T cells included a distinct CD103^{hi} population in young animals that was absent in adult mice (Fig. 5E). To further assess the magnitude of the contribution of RTEs to the CD103^{hi} population of naïve CD8⁺ T cells in

Fig. 6. Naïve CD8⁺ T cell preconditioning occurs in lymph nodes, but not spleens. (A) C57BL/6 mice were treated with FTY720 to block lymphocyte egress from all lymphoid tissues. After 4 weeks of continued treatment, naïve CD8⁺ T cells in LNs and spleen were analyzed for CD103 expression. Data are medians and replicates and are representative of six independent experiments. **(B)** CD103 expression in naïve CD8⁺ T cells in spleens and thymi of WT and *Lta*^{-/-} mice. Data are medians and replicates and are representative of two independent experiments. **(C)** Frequency of CD69⁺ CD103⁺ eT_{RM} cells in skin 4 weeks after “prime and pull” immune challenge through i.v. injection with OVA-expressing *L. monocytogenes* to produce a circulating T_{EFF} cell pool (“prime”) followed by DNFB⁺ treatment of ear skin (“pull”) of WT and *Lta*^{-/-} mice. Data are medians and replicates and are representative of four independent experiments. **(D)** CD103 expression of naïve CD8⁺ T cells from α V- Δ DC donors in the indicated SLOs of continuously FTY720-treated WT hosts 4 weeks after adoptive transfer. Data are medians and replicates and are representative of two independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 [two-tailed unpaired Student's *t* tests in (A) to (C), one-way ANOVA in (D)].



adult WT mice, we treated them with the functional SiP-receptor antagonist FTY720. This blocks lymphocyte tissue egress and thereby “traps” T cells within their respective tissue locations, including the thymus (37). If CD103^{hi} naïve CD8⁺ T cells were predominantly RTEs, this treatment would be predicted to produce a decrease in CD103 expression in SLOs. However, CD103 expression did not decrease, but further increased on naïve CD8⁺ T cells trapped in LNs. By contrast, CD103 expression decreased in cells trapped in the spleen (Fig. 6A). Thus, RTEs do not appear to make a large contribution to the CD103^{hi} naïve CD8⁺ T cell pool in adult mice. Furthermore, CD103 expression in peripheral T cells is preferentially induced and sustained in LNs, but not in the spleen. To test

this hypothesis in an independent model, we analyzed lymphotoxin- α -deficient (*Lta*^{-/-}) mice, which lack LNs (32). Naïve CD8⁺ T cells in these animals expressed low levels of CD103, comparable to those in α V-DC mice (Fig. 6B). Expression was, however, normal in *Lta*^{-/-} CD8 SP thymocytes and could be restored in splenic *Lta*^{-/-} naïve CD8⁺ T cells by exposure to TGF- β in vitro (Fig. 6B and fig. S6A). Thus, there was no cell-intrinsic defect obstructing TGF- β -driven induction of CD103. T_{EFF}-like cells did not accumulate in spleens of *Lta*^{-/-} mice, indicating that this feature of α V- Δ DC mice was not directly caused by a lack of exposure of naïve CD8⁺ T cells to TGF- β -activating α V⁺ DC in LNs (fig. S6B).

Immune responses to skin challenge are predicted to be impaired in *Lta*^{-/-} mice,

given their lack of LNs. Therefore, to assess eT_{RM} cell formation in these animals, we resorted to a “prime and pull” approach, whereby systemic T cell activation is combined with locally induced tissue inflammation to elicit T_{RM} cell formation (33, 34). Four weeks after intravenous (i.v.) injection with OVA-expressing *Listeria monocytogenes* and skin treatment of *Lta*^{-/-} mice with DNFB, the frequency of CD103⁺ skin eT_{RM} cells was reduced to levels comparable to those of DNFB-treated α V- Δ DC mice (Fig. 6C). Thus, low CD103 expression reflective of impaired naïve CD8⁺ T cell conditioning in *Lta*^{-/-} mice is also linked to a profound reduction in the ability to form eT_{RM} cells in skin upon immune challenge.

To further assess whether LNs draining different tissues varied in their capacity for T

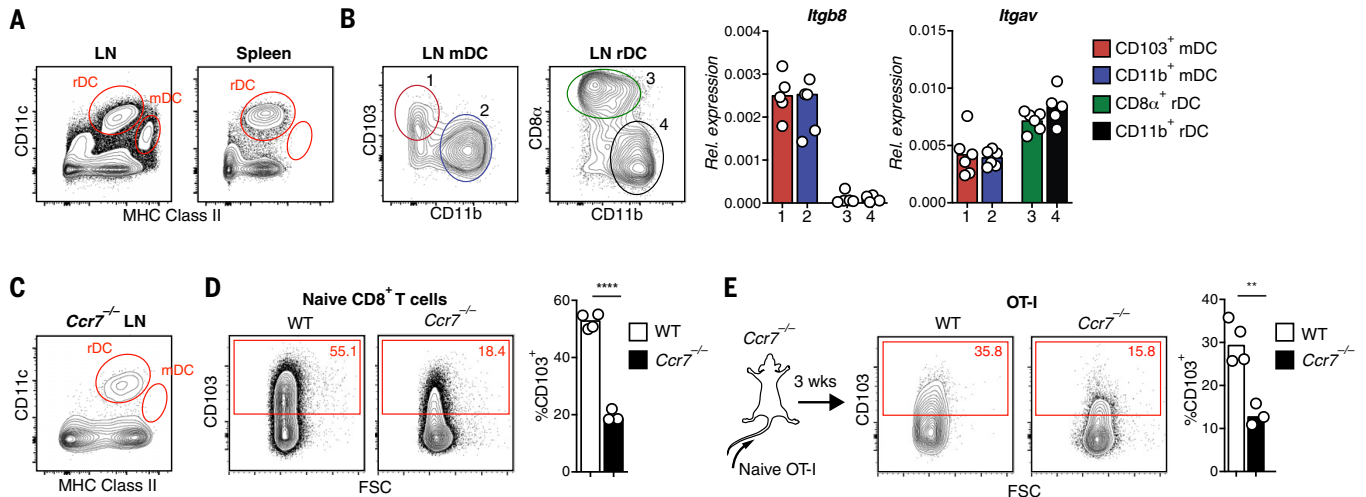


Fig. 7. $\alpha_V\beta_8$ -expressing migratory DCs precondition naïve CD8⁺ T cells for epithelial T_{RM} cell formation in skin. (A) CD11c^{int} MHC II^{hi} migratory DCs in LNs are absent in the spleens of C57BL/6 mice. (B) CD103⁺ and CD11b⁺ subsets of migratory DCs as well as CD8⁺ and CD11b⁺ subsets of resident DCs were purified by FACS and analyzed for expression of α_V and β_8 integrin mRNA by RT-qPCR. Data are means and replicates and are representative of two independent experiments. (C) CD11c^{int} MHC II^{hi} migratory DCs are absent

in LNs of *Ccr7*^{-/-} mice. (D) CD103 expression in naïve CD8⁺ T cells in pooled LNs and spleen of *Ccr7*^{-/-} mice. Data are medians and replicates and are representative of four independent experiments. (E) 10⁶ naïve OT-I T cells were adoptively transferred into *Ccr7*^{-/-} or C57BL/6 hosts. CD103 expression on naïve OT-I cells from pooled LNs and spleen was analyzed 3 weeks later. Data are medians and replicates and are representative of two independent experiments. ***p* < 0.01, *****p* < 0.0001 [two-tailed unpaired Student's *t* tests in (D) and (E)].

cell conditioning, we transferred CD103^{lo} naïve CD8⁺ T cells from α_V -ADC mice into FTY720-treated WT hosts. Four weeks later, CD8⁺ T cells in mesenteric LNs showed the most pronounced increase in expression of CD103, followed by CD8⁺ T cells in skin-draining LNs. By contrast, the induction of CD103 was moderate in lung-draining mediastinal LNs and absent in spleen (Fig. 6D). Thus, α_V -expressing DCs in LNs are specialized to precondition naïve CD8⁺ T cells to differentiate into eT_{RM} cells.

Preconditioning activity in $\alpha_V\beta_8$ -expressing migratory, but not resident, DCs

A key difference between LNs and spleen is the presence in the former of a CD11c^{int} MHC II^{hi} migratory DC (mDC) population (Fig. 7A) and (35). These cells continually traffic from nonlymphoid tissues to draining LNs via the lymph. Although both mDCs and resident DCs (rDCs), including CD11b⁺, CD103⁺, and CD8⁺ subsets, expressed α_V , only mDCs expressed the β_8 -integrin chain (Fig. 7B) to form the $\alpha_V\beta_8$ heterodimer that enables them to activate TGF- β (17).

Naïve CD8⁺ T cells expressed CD103 at similarly low levels in mice lacking β_8 integrin in DCs (*Cd11c*^{Cre} × *Itgb8*^{fl/f}, or “ β_8 -ADC” mice) as in α_V -ADC mice, although this defect was again less pronounced in very young mice with the highest frequencies of RTEs (fig. S6, C and D). Reduced eT_{RM} cell formation in the skin of β_8 -ADC mice further confirmed a role specifically for $\alpha_V\beta_8$ in T cell preconditioning (fig. S6E). However, the reduction in skin eT_{RM} cells was less severe than in α_V -

ADC mice, suggesting that either TGF- β -independent functions of DC-expressed α_V integrins, or TGF- β activation through other α_V heterodimers expressed by DC, such as $\alpha_V\beta_3$ and $\alpha_V\beta_5$, contribute to TGF- β activity on CD8⁺ T cells.

To further test if naïve T cell preconditioning through TGF- β depends on mDCs, we analyzed CCR7-deficient (*Ccr7*^{-/-}) mice, in which DC trafficking from peripheral tissues to draining LNs is defective and in which mDCs are absent from both the spleen and LNs (Fig. 7C) (36). Similar to the results in α_V -DC and *Lta*^{-/-} mice, CD103 expression was reduced on naïve CD8⁺ T cells in *Ccr7*^{-/-} mice (Fig. 7D). However, because *Ccr7*^{-/-} T cells may also be excluded from TGF- β -mediated preconditioning on the basis of their impaired ability to enter LNs, we transferred CCR7-sufficient OT-I T cells that can enter LNs into *Ccr7*^{-/-} hosts. These cells also lost CD103 expression (Fig. 7E), confirming the critical role for CCR7-dependent migration of α_V -expressing DCs from the skin to the LNs to precondition naïve CD8⁺ T cells to differentiate into eT_{RM} cells in the skin.

eT_{RM} cell preconditioning occurs through MHC I-dependent DC-T cell interactions

Naïve CD8⁺ T cell survival and responsiveness depends on noncognate, but MHC I-dependent, interactions with DCs (37). The question arose whether resting CD8⁺ T cells receive TGF- β signals during MHC-I-dependent physical interactions with α_V -integrin-expressing DCs, or if DCs produce a pool of active TGF- β

that is available indiscriminately to all T cells. We therefore generated mixed bone marrow chimeras (BMCs) from MHC I-deficient *B2m*^{-/-} and α_V -ADC donors to segregate expression of these two proteins on DCs and to test whether self-peptide-MHC I ligands and TGF- β -activating α_V integrins need to be coexpressed by the same DC for naïve T cell preconditioning to be effective. This strategy was only partially effective because about a third of DCs continued to coexpress MHC I and α_V , possibly as a result of transfer of membrane proteins between DCs (38). The remaining two-thirds were divided evenly between MHC I-expressing and α_V -expressing subsets (Fig. 8A). However, even a partial reduction in the frequency of DCs coexpressing MHC I and α_V resulted in a decrease in the frequency of CD103⁺ naïve CD8⁺ T cells, as compared to control BMCs (Fig. 8B). Accordingly, the capacity of OT-I cells transferred into *B2m*^{-/-}: α_V -ADC BMCs to form eT_{RM} cells in the skin upon vaccination was impaired (Fig. 8C). Thus, α_V -expressing DCs in LNs do not produce a public pool of active TGF- β available to all T cells but rather present it to naïve CD8⁺ T cells discretely during MHC I-dependent interactions.

Discussion

In this study, we show that MHC I-dependent interactions with DCs expressing TGF- β -activating α_V integrins epigenetically precondition naïve CD8⁺ T cells during the steady state to efficiently form epidermal T_{RM} cells upon foreign antigen encounter. This activity

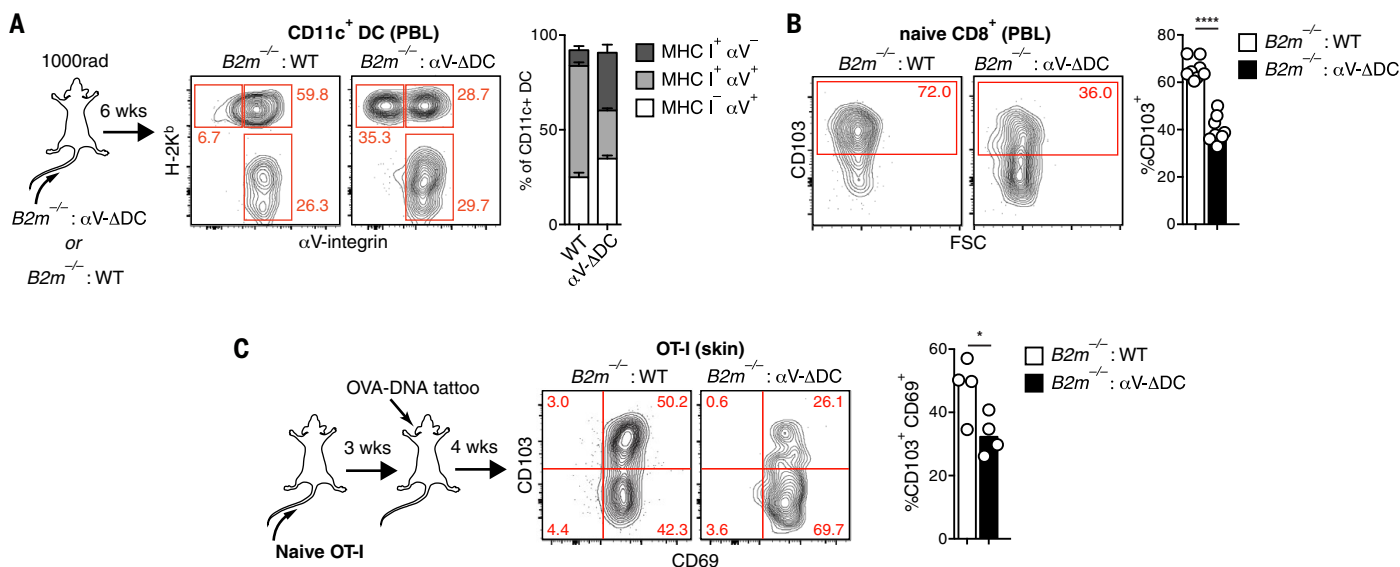


Fig. 8. eTRM cell preconditioning of naive CD8⁺ T cells occurs through homeostatic MHC I-dependent DC interactions. (A) Lethally irradiated CD45.1 congenic mice were injected with 1:1 mixtures of either WT or αV-ADC and MHC I-deficient B2m^{-/-} bone marrow cells. Eight weeks later, the expression of H-2K^b and αV on CD45.1⁺ CD11c⁺ cells in peripheral blood was assessed to determine the frequency of cells that express either one of both proteins. Data are means ± SEM (*n* = 8 mice for WT and *n* = 6 mice for αV-ADC) and representative of four independent experiments. (B) CD103 expression on naive CD8⁺ T cells in the peripheral blood of

αV-ADC: B2m^{-/-} mixed BMCs, in which expression of αV and MHC I on DCs is segregated. Data are medians and replicates and are representative of two independent experiments. (C) 10⁶ naive OT-I cells were adoptively transferred into BMCs. Three weeks later, mice were vaccinated by ear tattoo with OVA-encoding plasmid DNA. After an additional 4 weeks, CD69 and CD103 expression was assessed on transferred OT-I T cells in skin. Data are medians and replicates and are representative of two independent experiments. **p* < 0.05, *****p* < 0.0001 [two-tailed unpaired Student's *t* tests in (B) and (C)].

is restricted to migratory DCs found in LNs, but not in the spleen, delineating an unanticipated division of labor between these lymphoid tissues.

DCs are known to imprint specific migratory patterns in the T cells they activate. For instance, DCs in skin-draining LNs induce the preferential expression of homing molecules for entry into skin, whereas DCs in mesenteric LNs elicit tropism for the small intestine (39–41). eTRM cell preconditioning is distinct in that it already occurs during immune homeostasis, whereas imprinting for tissue-selective homing occurs during T cell priming. Furthermore, αV-integrin-mediated exposure to TGF-β is similar in skin and mesenteric LNs, judging from comparable induction of CD103 in naive T cells at both sites. Preconditioning was, however, less pronounced in mediastinal LNs, raising the possibility that migratory DCs from different tissues possess varying capacities for TGF-β activation in draining LNs.

It is perhaps counterintuitive that the propensity for eTRM cell formation is conferred upon T cells not during antigenic priming in LNs, but rather earlier, under resting conditions. As a result, naive CD8⁺ T cells, by transiently retaining the preconditioned state during recirculation, remain endowed with the potential to form eTRM cells even when activated in other SLOs, such as the spleen.

In this scenario, the propensity for eTRM cell differentiation does not result from activation at a specific anatomical site, but may instead be restricted to a subset of a heterogeneous naive T cell pool that is competent to receive TGF-β signals from αV-integrin-expressing mDCs at steady state. Although the naive T cell repertoire is generally viewed as functionally homogeneous and uncommitted to any particular memory or effector fate (42), the precedent for such heterogeneity comes from studies on the developmental history of individual naive T cells (43) or their self-reactivity (44). In both studies, subsets were defined with an enhanced ability to rapidly differentiate into short-lived effector cells. Similarly, cell-intrinsic properties that remain to be defined may render a subset of naive CD8⁺ T cells, identifiable by expression of the CD103 integrin, competent for TGF-β-mediated preconditioning for a future eTRM cell fate.

It has long remained unclear how the multitude of biological functions of a widely secreted cytokine like TGF-β on various cell types could be regulated and coordinated. Recently, LRRC33 was identified as a previously unknown TGF-β milieu protein expressed on microglia, which enables highly localized TGF-β activity in the central nervous system in coordination with αVβ8-integrins on glial cells (45). Although no milieu proteins expressed by naive CD8⁺ T cells have yet been

identified, our observation that exposure to activated TGF-β is limited to cells that engage in homeostatic, MHC-I-restricted physical interactions with αV-expressing DCs further illustrates an important general principle: namely, that the potent biological activity of this pleiotropic cytokine can be restricted to individual cells through a two-cell mechanism and thereby “privatized.”

It will be of interest to determine if the DC-driven, TGF-β-dependent preconditioning of naive CD8⁺ T cells in LNs can be limiting in situations where maximal formation of eTRM cells would be desirable (e.g., during vaccination). In such cases, transiently optimizing local or systemic TGF-β activity in preparation for vaccine administration, ideally targeted to CD8⁺ T cells in SLOs, may allow for the manipulation of T cell memory differentiation to improve vaccine-induced protection. Conversely, approaches to disrupt naive T cell preconditioning might serve to attenuate de novo eTRM cell formation and aid in the treatment of diseases such as psoriasis, in which eTRM cells play pathogenic roles.

Materials and methods

Mice

Mice with either floxed αV alleles (*Itgav*^{fl/fl}) or floxed β8 alleles (*Itgb8*^{fl/fl}) were previously described (15, 17) and crossed to CD11c^{Cre} BAC-transgenic mice (16) obtained from The Jackson

Laboratory. C57BL/6J, CD45.1 or Thy1.1 congenic C57BL/6J, OT-I $\times$ *Tcr α ^{-/-}*, *Cd103^{-/-}*, *B2m^{-/-}*, *Ccr7^{-/-}*, and *Lta^{-/-}* mice were purchased from The Jackson Laboratory and further bred in-house. Animals were housed in specific pathogen-free facilities at the Massachusetts General Hospital (MGH) and all experimental studies were approved and performed in accordance with guidelines and regulations implemented by the MGH Institutional Animal Care and Use Committee (IACUC).

Irradiation bone marrow chimeras

Recipient mice were i.p. injected with 200 μ g of α -NK1.1 monoclonal antibodies (mAbs) (clone PK136, BioXCell) 1 day prior to irradiation to transiently deplete NK cells and enhance engraftment of *B2m^{-/-}* bone marrow. Bone marrow was dislodged from femurs and tibiae of donor mice by flushing with 10 ml of phosphate-buffered saline without Ca^{2+} / Mg^{2+} ("PBS" hereafter) and filtered once through a 40- μ m filter. Cells from either WT or α V-ADC mice were mixed at a 1:1 ratio with cells from *B2m^{-/-}* mice and resuspended at 5×10^7 total cells/ml. Recipients were irradiated at 1000 rad (cesium) in a rotating chamber and injected retro-orbitally with 5×10^6 total donor bone marrow cells/mouse in 100 μ l of PBS. Mice were provided sulfamethoxazole in their drinking water for 4 weeks after irradiation.

DNA vaccination, mechanical skin irritation, and DNFB treatment

For DNA vaccination against OVA, the 6162-base pair (bp) expression plasmid pOD CAGGS was kindly provided by J. J. Moon. pOD CAGGS is derived from pCAGGS (46) and uses the CAG promoter to drive expression of full length chicken ovalbumin fused on its N terminus to the leader sequence of the H-K^b α -chain (MVPCTLLLLLAAALAPTQTRA) and fused on its C terminus to 44 amino acids of the transmembrane domain of H-2D^b (HEGLPEPLTLRWEPPPSTDSYMVIVAVLGVLGAMAIIGAVVAFV) to achieve membrane incorporation. Mice were anesthetized using isoflurane. Ear or flank skin was shaved and epilated using Nair hair removal cream. A droplet of H₂O containing 3 μ g of pOD CAGGS DNA was then tattooed into the skin using a sterile disposable 11-needle bar mounted on a rotary tattoo device (Biotouch), as previously described (20). To create a transient inflammatory reaction through mechanical skin irritation alone, plasmid DNA was omitted from this procedure. Alternatively, mice were treated with 10 μ l of 0.5% DNFB in a 4:1 acetone:olive oil emulsion to inflame ear skin.

HSV-OVA infection

HSV-OVA was obtained from T. Gebhardt (University of Melbourne). For epicutaneous infection by scarification a small (~5 mm²)

area of skin overlying the upper pole of the spleen was abraded with 20 strokes of 150-grit sandpaper attached to the end of a pencil (47). Then, 10⁶ plaque-forming units (PFU) of HSV-OVA in 10 μ l HBSS were applied to the abraded site, and mice were banded for 2 days as described (47, 48). To measure viral titers, a 1-cm² area of skin surrounding the infection site was harvested into Dulbecco's minimum essential medium (DMEM), cut into small pieces, and snap frozen in a dry ice–70% ethanol bath. The snap-frozen tissue was thawed at 37°C in a water bath and homogenized in gentleMACS M tubes in a total volume of 2 ml using the RNA_01 protocol. Samples were centrifuged for 5 min at 500 $\times$ g to remove debris and aggregates and supernatants serially diluted (range, 10⁻¹ to 10⁻⁶) in serum-free Vero Medium (DMEM, 1% HEPES, 1% GlutaMAX). Serially diluted virus suspension (200 μ l) was added to Vero cells grown to 90% confluency in 24-well plates (in duplicate for each sample and dilution). After 1 hour of incubation at 37°C with gentle rocking of the plate every 10 to 15 min for even distribution of virus, viral dilutions were aspirated, cells lightly washed with PBS, and cultured for 2 days at 37°C in 1 ml/well of incubation medium [DMEM, 1% HEPES, 1% GlutaMAX, 1% penicillin–streptomycin, 5% (nonfetal) bovine serum, 7.5 μ g/ml human IgG]. Cells were then washed with PBS, stained with 1 ml of crystal violet solution (20% EtOH, 80% H₂O, 0.5 g/100 ml of crystal violet powder) for 15 min, washed with H₂O, and dried overnight. Plaques were counted manually.

Prime and pull immune challenge

The attenuated OVA-expressing *L. monocytogenes* strain Lm-OVA Δ actA was provided by J. J. Moon (MGH) and was grown in brain heart infusion (BHI) medium containing 34 μ g/ml of chloramphenicol to an absorbance of ~0.1 at 600 nm. Lm-OVA Δ actA (2×10^7 CFU) were then injected intravenously into mice to induce a systemic T_{EFF} cell response ("Prime"). Four days after infection, mice ears were treated with DNFB, as described above, to produce skin inflammation ("Pull") and enable seeding by T_{RM} cells from the circulating pool of T_{EFF} cells.

Adoptive T cell transfers, in vitro activation of T cells, and T cell depletion

Naïve CD8⁺ T cells were purified from LN and spleen single-cell suspensions by immunomagnetic negative cell selection using the Miltenyi naïve CD8⁺ T cell isolation kit and adoptively transferred into sex-matched recipients by retro-orbital injection. For the in vitro activation of T cells, OT-I $\times$ *Tcr α ^{-/-}* splenocytes were pulsed with 1 μ M SIINFEKL peptide (New England Peptide) in 1 ml of T cell medium [RPMI, 10% fetal calf serum

(FCS), 1% HEPES, 1% sodium pyruvate, 1% GlutaMAX, 1% nonessential amino acids, 55 μ M 2-mercaptoethanol] for 1 hour at 37°C, diluted in 9 ml of T cell medium, and cultured at 37°C in 5% CO₂. Two days later, 20 ng/ml of IL-2 was added and cell density was maintained at 10⁶ cells/ml. OT-I cells were adoptively transferred on day 5 after activation by retro-orbital injection.

For T cell depletion, one dose of anti-Thy1.2 mAbs (3 μ g, clone 30H12) was injected i.v.

FTY720 treatment

Mice received intraperitoneal injections of 1 mg/kg BW FTY720 (fingolimod) (Sigma-Aldrich) in 150 μ l of H₂O every 2 to 3 days.

Naïve T cell treatment with TGF- β 1

CD44^{lo} naïve CD8⁺ T cells were purified from LNs and spleens by immunomagnetic negative cell selection using the Miltenyi naïve CD8⁺ T cell isolation kit. Cells were cultured in 96-well flat-bottom plates at a density of 2×10^6 cells/ml in serum-free XTVIVO10 medium supplemented with 100 ng/ml of rmIL-15 and 5 ng/ml of rmIL-7 (BioLegend). Titrated amounts of acid-activated recombinant mouse TGF- β 1 (Cell Signaling) were added as indicated, and cells were cultured at 37°C in 5% CO₂ for 72 hours before analyzing CD103 surface expression.

Isolation of cells from tissues and staining for flow cytometry

For cell isolations from non-lymphoid tissues, mice were i.v. injected with 3 μ g of Alexa Fluor 700- or fluorescein isothiocyanate (FITC)-labeled α -CD45.2, or PE-Cy7-labeled α -CD8 β antibody 3 min prior to euthanasia to label intravascular leukocytes for exclusion from analysis. All organs were harvested into ice-cold FACS (fluorescence-activated cell sorting) buffer [PBS with 0.5% bovine serum albumin (BSA) and 2 mM EDTA].

Skin was processed as previously described (49). Briefly, separated dorsal and ventral halves of ear skin or flank skin were minced into small pieces and placed in 2.5 ml of digest buffer A [DMEM; 2% fetal bovine serum (FBS); 1% HEPES; 25 U/ml collagenase IV] or digest buffer B [DMEM; 2% FBS; 1% HEPES; 125 μ g/ml liberase TM (Sigma-Aldrich); 0.5 mg/ml hyaluronidase Type I-S from bovine testes (Sigma-Aldrich)] for 1 hour at 37°C under agitation, then quenched with 10% FBS and 1.5 mM EDTA and blended using a gentleMACS tissue blender (Miltenyi, C tubes, m_impTumor_01 protocol).

Spleens, thymi, and LNs were minced and passed through a 40- μ m cell strainer. Red blood cells were lysed with ACK lysis buffer as necessary. For the isolation of DCs, minced spleens and LNs were digested in digest buffer A for 20 min under agitation at 37°C before passing through a 40- μ m cell strainer.

Cell surface epitopes were stained in FACS buffer in the dark at 4°C for 15 min, followed by staining with fixable viability dye (Zombie Dyes, BioLegend) at room temperature for 15 min. For detection of intracellular epitopes, cells were fixed and permeabilized (eBioscience Fixation/Permeabilization kit) and stained with antibodies for 30 min in the dark at room temperature. All antibodies used are listed in table S2.

Histology

Mice were epilated (Nair) and excised ear skin was fixed for 15 min at room temperature in 4% paraformaldehyde (PFA) (freshly diluted from 16%), washed six times for 30 min each time in PBS, and stored overnight in 30% sucrose. Eighteen hours later, tissues were immersed in OCT, snap-frozen in a dry ice–2-methylbutane bath, and stored at –80°C until sectioning. Frozen cross-sections (10 to 20 µm) were air-dried at room temperature for 5 min and loaded into a Shandon Immunostaining Chamber with 1 ml of PBS. Tissues were fixed again with 100 µl 4% PFA for 10 min, washed with 1.2 ml of PBS, and then washed with 200 µl of 0.3% PBST. Tissues were then blocked with 100 µl of blocking buffer (5% normal donkey serum, 1% BSA, 2% cold-water-fish gelatin, 0.3% Triton X-100 in PBS) for 30 min at room temperature. Sections were incubated overnight at 4°C with primary antibodies (in blocking buffer, 100 µl total per slide), washed with 1 ml 0.3% PBST, and incubated with streptavidin for 1 hour at room temperature. Slides were washed with 1 ml of 0.3% PBST and then 2 ml of PBS. Slides were then mounted with 100 µl of DAPI fluoromount (Southern Biotech) and cured overnight in the dark at room temperature. Images were acquired on a Zeiss LSM 800 confocal microscope using a 20×/0.8 NA (numerical aperture) dry lens and processed using Imaris Software (Bitplane) and ImageJ software (NIH).

The density of immune cells in skin was determined by manual counting and extrapolation of the number of cells per cm² skin surface on the basis of the number of cells per section and the skin surface represented by the section (10-µm thickness × measured length of the epithelium in the field of view). Nonconsecutive sections were analyzed for quantification to avoid duplicate counts of cells overlapping between sections.

Separate tissues (ear skin and large intestine) from 7-week-old mice were fixed overnight in neutral-buffered formalin (Sigma-Aldrich), paraffin-embedded, sectioned, and stained with hematoxylin and eosin.

ELISA of serum immunoglobulins

Serum immunoglobulins were measured as previously described (50). Immulon 2HB microtiter plates (DYNEX) were coated with 10 µg/ml

of goat anti-mouse Ig (Southern biotech) in PBS at 4°C overnight. Plates were then blocked with 1% BSA in PBS and incubated with serial dilutions of serum samples in PBS. Specific Ig isotypes were detected using alkaline phosphatase-conjugated isotype-specific antibodies (Southern Biotech). Alkaline phosphatase activity was developed with disodium *p*-nitrophenyl phosphate substrate (Southern Biotech). Antibody concentrations were calculated as titers relative to purified Ig standards (Southern Biotech).

Polymerase chain reaction (PCR) analysis of floxed *Itgav* alleles

Five thousand cells of each type were purified by FACS, and genomic DNA was extracted using the Arcturus PicoPure DNA Extraction Kit. DNA was amplified for 35 cycles with an annealing temperature of 57°C using Q5 High Fidelity DNA Polymerase and the following primers: *Intav4avf*: 5'-TTCAGGACGGCACA-AAGACCGTTG-3'; and *Intav5b3*: 5'-CACAA-ATCAAGGATGACCAAACTGAG-3'. WT *Itgav* and floxed *Itgav* allele products were sized 150 and 400 bp, respectively, whereas the recombined floxed *Itgav* allele yielded no product. The ratio of WT *Itgav* to floxed *Itgav* product was determined for each sample and normalized to the ratio in a tail sample of an *Itgav*^{fl/wt} control animal not expressing Cre recombinase, to determine the degree of deletion in each cell type.

Preparation of genomic material and ATAC-seq data analysis

LN CD44^{lo} naïve CD8⁺ T cells (3 × 10⁴ per replicate) were purified by FACS into PBS containing 10% FBS in DNA loBind Eppendorf tubes. Pelleted cells were lysed in 50 µl of reaction mix (25 µl of 2× tagment DNA (TD) buffer, 2.5 µl of Tn5 enzyme, 0.25 µl of 2% digitonin, and 22.25 µl of nuclease-free water). The mix was incubated at 37°C for 30 min with agitation at 300 rpm. DNA was purified using a QIAgen MinElute Reaction Cleanup kit and Nextera sequencing primers ligated using PCR amplification. Agencourt AMPure XP bead cleanup (Beckman Coulter/Agencourt) was used post-PCR and library quality was verified using a TapeStation machine. Samples were sequenced on an Illumina HiSeq 2000 sequencer using paired-end 5' bp reads.

For analysis of sequences, first, adapters were trimmed using AdapterRemoval (v. 2.2.1a) (51). Second, the paired-end ATAC-seq were aligned to the mouse reference genome (mm10) using Bowtie2 (2.3.4.1) (52) with the following parameters: –no-discordant–no-unal–no-mixed –X 2000. Third, the reads were shifted (Tn5 insertion) after removing the reads from mitochondrial DNA and duplicated reads.

ATAC-seq peak regions of the pooled sample were called using HOMER (v4.9.1) (53)

with the following parameters: –region –size 500 –minDist 50 –o auto –tbp 0. Then, the fragment counts for each peak region for each sample were obtained using bedtools (v2.24.0) (54). Differentially accessible peak regions between conditions using the two replicates per condition (adjusted *p* value ≤ 1 × 10^{–5}) were called using DESeq (v1.30.0) (55) using the “pooled” dispersion estimation with the “local” fit. Then, overlapping differentially accessible peak regions (adjusted *p* value ≤ 1 × 10^{–5}) per condition were merged using bedtools. For each merged peak region, as illustrated in Fig. 4A, we associate adjusted *p* and log₂ fold-change values using the adjusted *p* and log₂ fold-change values of the region of smallest adjusted *p* value (among the regions prior to merging).

ATAC-seq coverage tracks were generated using deepTools (v3.0.2) (56). The size factors estimated by DESeq were used to normalize ATAC-seq coverage tracks visualized in Figs. 4D and fig. S4A.

HOMER was used to annotate the merged differentially accessible regions per condition and to calculate distances from DARs to the closest transcription start sites to generate Fig. 4B. Additionally, HOMER was used to find enriched motifs in the merged differentially accessible regions of each condition with the following parameters: –size given –mask –nomotif, and using the default motif collection (364 motifs). To generate Fig. 4C, we used the motif family information included in the HOMER motif database and discarded the families with the “?” symbol in their identifier. Moreover, we separated the KLF motifs from the Zf family: The KLF family contains all the KLF motifs and the Zf (others) family contains all other Zf motifs.

The Genomic Regions Enrichment of Annotations Tool (GREAT) was used for gene ontology and molecular signature enrichment analysis (57). DARs were inputted into the GREAT and gene associations were defined by “basal plus extension” (basal as 5 kb upstream of and 1 kb downstream from the TSS, and extension to estimate gene regulatory regions 1 Mb upstream and downstream of the TSS.)

Reverse transcriptase quantitative PCR (RT-qPCR)

Dendritic cells from each indicated subset (2 × 10⁵ per subset) were purified by FACS into TriZOL for RNA extraction and RNA was further purified using RNeasy Plus Micro or Mini Kit (Qiagen). RNA was reverse transcribed using a High Capacity cDNA Transcription Kit (Life Technologies) and RT-qPCR was performed using SYBR Green detection (Roche LightCycler 480 kit). Primer sequences used for amplification are as follows: *Itgav* (forward: 5'-CGGGTCCCGAGGGAAGTTA-3'; reverse: 5'-TGGATGAGCATTACATTGAG-3')

and *Itgb8* (forward: 5'-AGTGAACACAATAGATGTGGCTC-3'; reverse: 5'-TTCTCTGATCCACCTGAACAAAA-3').

Statistical analysis

Two-tailed, paired or unpaired Student's *t* tests (for normally distributed data) or Mann-Whitney *U* tests (for non-normally distributed data) was used for comparisons between two groups. One-way analysis of variance (ANOVA) test was used for comparisons between multiple groups. The two-sample Kolmogorov-Smirnov test was used to compare cumulative distributions. All statistical tests were performed with GraphPad Prism software, and *p* < 0.05 was considered statistically significant.

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experiments; V.M. analyzed the data; A.L.-H. and M.A.T. generated mice; A.L.-H., K.L.J., and A.D.L. made important conceptual contributions; and V.M. and T.R.M. designed the experiments and wrote the manuscript. **Competing interests:** The authors declare no competing interests. **Data and materials availability:** ATAC-seq data are available under GEO accession number GSE133504. All other data needed to evaluate the conclusions in this paper are available in the main text or the supplementary materials.

SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/366/6462/eaav5728/suppl/DC1
Figs. S1 to S6
Tables S1 and S2
[View/request a protocol for this paper from Bio-protocol.](#)

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RESEARCH ARTICLE

STRUCTURAL BIOLOGY

Architecture of human Rag GTPase heterodimers and their complex with mTORC1

Madhanagopal Anandapadamanaban¹, Glenn R. Masson¹, Olga Perisic¹, Alex Berndt^{1*}, Jonathan Kaufman¹, Chris M. Johnson¹, Balaji Santhanam¹, Kacper B. Rogala², David M. Sabatini^{2,3,4,5,6}, Roger L. Williams^{1†}

The Rag guanosine triphosphatases (GTPases) recruit the master kinase mTORC1 to lysosomes to regulate cell growth and proliferation in response to amino acid availability. The nucleotide state of Rag heterodimers is critical for their association with mTORC1. Our cryo-electron microscopy structure of RagA/RagC in complex with mTORC1 shows the details of RagA/RagC binding to the RAPTOR subunit of mTORC1 and explains why only the RagA_{GTP}/RagC_{GDP} nucleotide state binds mTORC1. Previous kinetic studies suggested that GTP binding to one Rag locks the heterodimer to prevent GTP binding to the other. Our crystal structures and dynamics of RagA/RagC show the mechanism for this locking and explain how oncogenic hotspot mutations disrupt this process. In contrast to allosteric activation by RHEB, Rag heterodimer binding does not change mTORC1 conformation and activates mTORC1 by targeting it to lysosomes.

The mechanistic target of rapamycin complex 1 (mTORC1) is a Ser/Thr protein kinase complex that integrates signals from nutrient availability, energy, and growth factors to regulate cell growth, proliferation, and metabolism (1). Up-regulation of mTORC1 is associated with many diseases such as cancer, type 2 diabetes, and defects in neurodevelopment (1, 2). The mTORC1 complex is a dimer of mTOR/RAPTOR/mLST8 heterotrimer (3). The mTORC1 kinase activity is tightly regulated by two classes of small GTPases, Rags and RHEB, both of which are necessary for activation (4, 5). In response to the abundance of nutrients, particularly amino acids, active Rag heterodimers bind the RAPTOR subunit of mTORC1 to recruit it to lysosomes (6–8), where mTORC1 can be allosterically stimulated by growth factor-activated RHEB (3, 9–12). Unlike RHEB, which carries a C-terminal farnesylation that weakly associates it to a variety of membranes (13, 14), Rags have no lipid modification. Instead, they associate with the heteropentameric Regulator complex, which has myristoyl and palmitoyl modifications at the N terminus of its LAMTOR1 subunit that localize it to lysosomes (15, 16). Recurrent oncogenic mutations in RagC enhance its association with mTORC1, leading to increased mTORC1 signaling (17–19).

Both Rags and RHEB are members of the Ras-like superfamily of GTPases. However, unlike most members, Rags are obligate heterodimers, with RagA or RagB pairing with RagC or RagD (20). Analysis of the composite genome of *Lokiarchaeum* revealed that Rag GTPases have an archaeal origin closely related to the Arf family of Ras-like GTPases that are involved in vesicular sorting (21), but the mechanistic implication of this similarity was not clear.

Rag heterodimers have four possible nucleotide-binding states but are active for mTORC1 binding only when RagA or RagB is guanosine triphosphate (GTP)-bound and RagC or RagD is guanosine diphosphate (GDP)-bound (6, 7, 22). The GTPase domains communicate so that binding of GTP by one subunit inhibits GTP binding and induces GTP hydrolysis by the other subunit (22). The nucleotide states of Rags are regulated by GTPase-activating proteins (GAPs) such as GATOR1 and folliculin (23–25) and guanine nucleotide exchange factors (GEFs) such as SLC38A9 and Regulator (26, 27).

To elucidate how human Rags interact with mTORC1 and how RagC mutations activate mTORC1, we determined the structures and dynamics of RagA/RagC complexes in isolation and bound to mTORC1. The structures revealed nucleotide-dependent conformational changes in Rags that are required for mTORC1 binding, enabling us to understand the mechanism by which oncogenic Rag mutations facilitate association with mTORC1.

HDX-MS shows that RagA/RagC protects the α -solenoid of RAPTOR

To map RagA/RagC interactions with the RAPTOR subunit of mTORC1, we carried out

hydrogen/deuterium exchange mass spectrometry (HDX-MS). For this we used RagA-Q66L_{GTP}/RagC-T90N_{GDP} containing mutations that increase mTORC1 association (6, 7, 17, 19). The RagA-Q66L switch II mutation (fig. S1) (28) impairs GTP hydrolysis and is a potent activator of mTOR signaling, with mice bearing this mutation dying within 1 day of post-natal life (29). The RagC-T90N mutation binds only GDP and is the most frequent and potent oncogenic mutation in RagC (17, 19).

RagA/RagC heterodimers were monodisperse (fig. S2A) and formed a 1:1:1 complex with RAPTOR (fig. S2, B and C). Most RAPTOR peptides that showed decreased HDX upon RagA/RagC binding mapped to a contiguous surface in the region of residues 541 to 678, encompassing three adjacent helical repeats of the RAPTOR α -solenoid (Fig. 1A, fig. S3, and table S1). There was also reduced HDX in the insertion before the last two helices of the RAPTOR α -solenoid (peptides 760 to 780 and 805 to 812) and in the WD40 domain itself. On the Rag side of the interface, RagA switch I was protected from HDX by RAPTOR (Fig. 1B), whereas RagC switches were not (Fig. 1C and table S1). To understand the context of the HDX-MS-measured dynamics, we determined the structures of RagA/RagC heterodimers both free and bound to mTORC1.

High-resolution crystal structures of active RagA/RagC heterodimers

HDX-MS identified RagC residues 1 to 34 as highly flexible (fig. S2D). For crystallization, we truncated this region, producing a variant RagC(34-399) that bound RAPTOR the same as full-length RagA/RagC (fig. S2E) and thereby enabled us to determine high-resolution crystal structures of RagA/RagC.

The crystal structure of RagA-Q66L/RagC(34-399)-T90N at 2.6 Å resolution showed a compact arrangement of C-terminal CRD domains that mediate heterodimerization and N-terminal GTPase domains (Fig. 1D and table S2). The overall fold of the GTPase domains is similar to other Ras-like GTPases, with conserved loops (G1 to G5 motifs) that engage bound nucleotides, as well as regions that change conformation depending on whether GTP or GDP is bound, known as switches (30). RagA is bound to GTP and has Mg²⁺ associated with the GTP γ -phosphate, whereas RagC is bound to GDP and has no bound Mg²⁺ (Fig. 1, E and F). Whereas RagA has switch I, interswitch, and switch II ordered (Fig. 1D and fig. S1), the RagC-T90N_{GDP} GTPase domain has no density for all of switch I and interswitch strand β 2 (residues 84 to 105) and all of switch II (116 to 130). The C-terminal CRDs have a roadblock fold consisting of a central five-stranded antiparallel β sheet sandwiched between two α -helical layers (31).

¹MRC Laboratory of Molecular Biology, Cambridge CB2 0QH, UK. ²Whitehead Institute for Biomedical Research, Cambridge, MA 02142, USA. ³Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142, USA. ⁴Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. ⁵Koch Institute for Integrative Cancer Research, Cambridge, MA 02139, USA. ⁶Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA.

*Present address: Astex Pharmaceuticals, Cambridge CB4 0QA, UK.
†Corresponding author. Email: rlw@mrc-lmb.cam.ac.uk

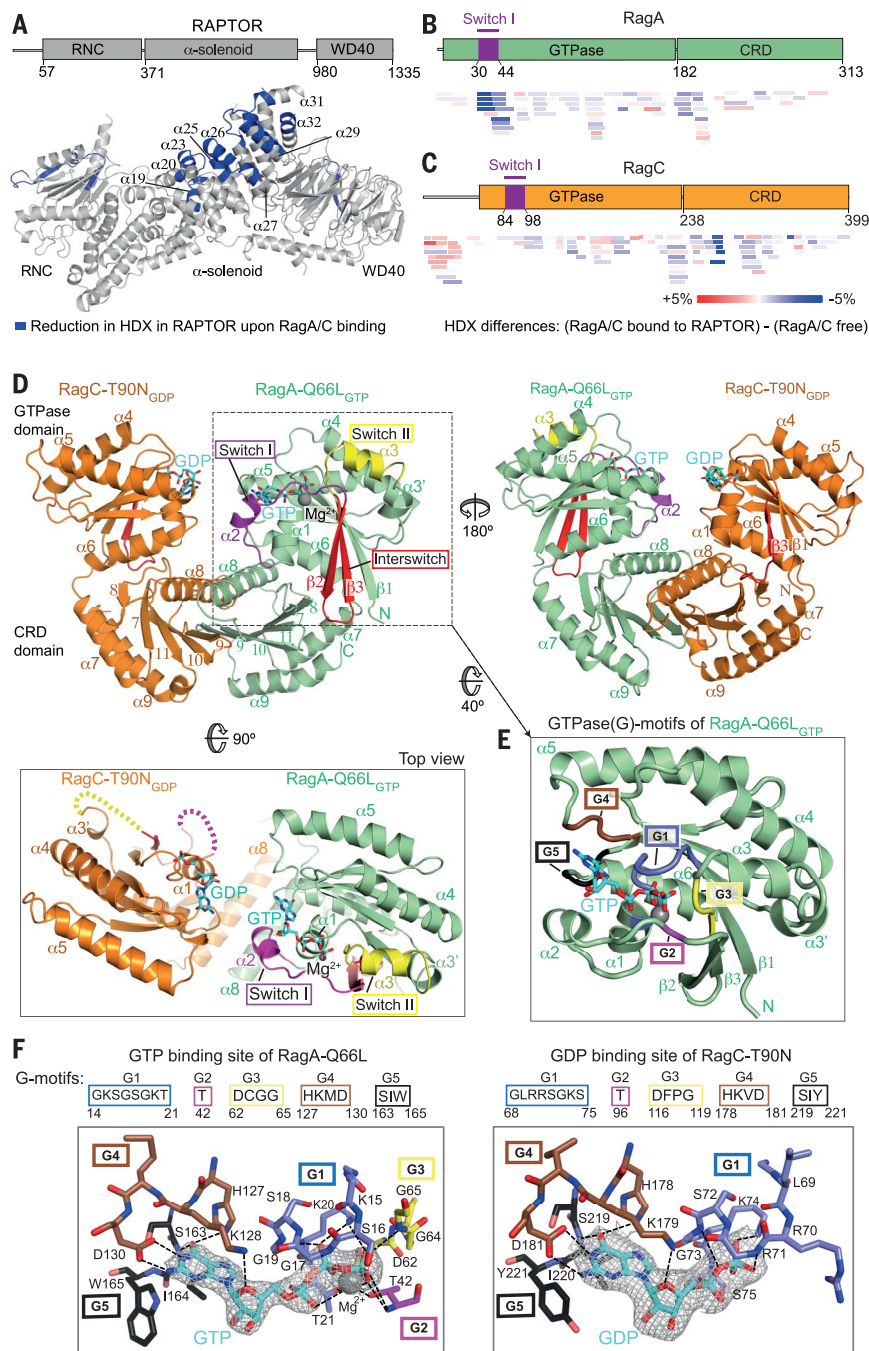


Fig. 1. The crystal structure of a RagA/RagC heterodimer and HDX-MS analysis of its interaction with RAPTOR. (A) HDX-MS-identified regions protected from HDX in the RAPTOR/RagA-Q66L_{GTP}/RagC-T90N_{GDP} complex. Decreases in HDX (blue) of RAPTOR upon RagA/RagC binding are depicted on the RAPTOR structure (from PDB ID 6BCX). (B and C) Differences in HDX for RagA-Q66L_{GTP} (B) and RagC-T90N_{GDP} (C) upon RAPTOR binding for all the peptides at 0.3 s in D₂O. Decreases in HDX are depicted in shades of blue, increases in shades of red. (D) The crystal structure of RagA-Q66L_{GTP}/RagC(34-399)-T90N_{GDP}, highlighting the ordered switches in RagA_{GTP} compared with disordered switches in the RagC-T90N_{GDP} oncogenic mutant. (E) The conserved G motifs of RagA-Q66L_{GTP} that make up the nucleotide binding pocket. (F) The 2mF_o - DFC map (contoured at 1.2 σ) for the GTP of RagA and the GDP of RagC together with the putative H bonds that they make to the G motifs. These are much more extensive for GTP than for GDP. Amino acid abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

The whole complex has a pseudo-two-fold symmetry, with the two GTPase domains close to each other and their switches on opposite faces of the complex (Fig. 1D).

The cryo-EM structure of mTORC1 bound to RagA/RagC

To understand how active RagA/RagC interacts with intact mTORC1, we used cryo-electron microscopy (cryo-EM). In order to stabilize mTORC1 bound to RagA-Q66L_{GTP}/RagC-T90N_{GDP}, we used chemical cross-linking and expressed the RagA/RagC heterodimer with RagC fused to another mTORC1-binding protein, PRAS40, which is largely disordered (fig. S4 and table S3). Using this mTORC1-RagA/RagC complex (fig. S5A), we generated a final reconstruction of mTORC1 at 4.1 Å resolution (figs. S5 and S6A and table S4). This reconstruction showed extra density adjacent to the α -solenoid region of RAPTOR (fig. S6A) that HDX-MS identified as the RagA/RagC binding site. The TOS motif from PRAS40 (fused to RagC) contacts a groove between the RAPTOR N-terminal conserved (RNC) and the α -solenoid of RAPTOR, as also observed by HDX-MS (fig. S4, C to E).

Focused classification with signal subtraction (32) showed that about 9.5% of particles were bound to RagA/RagC, corresponding to 90,809 particles, and reconstruction of the mTORC1-RagA-Q66L_{GTP}/RagC-T90N_{GDP} complex at 5.5 Å resolution revealed density for the RagA/RagC into which we could readily fit our high-resolution RagA/RagC crystal structure (Fig. 2 and fig. S6, B to D). RagA/RagC interacts with the convex surface of the RAPTOR α -solenoid (Fig. 3A). The GTPase-containing ends of the horseshoe-shaped RagA/RagC heterodimer are closest to the RAPTOR α -solenoid, with the CRDs pointing away from RAPTOR (Fig. 2, B and C). The RagA GTPase domain makes much more extensive RAPTOR contacts than RagC, and the interface agrees with our HDX-MS analysis (Fig. 3, A and B). The overall conformation of mTORC1 bound to RagA/RagC heterodimers is nearly identical to the conformation of apo-mTORC1 (3).

Three helices from RAPTOR (α 24, α 26, and α 29) in the region of residues 546 to 650 of the RAPTOR α -solenoid make an extensive network of interactions with switch I and interswitch strand β 2 of RagA-Q66L_{GTP} (Fig. 3A). The GTPase domain of RagC_{GDP} forms limited interactions with RAPTOR, including a contact of RagC-Asp¹⁸⁵ at the N terminus of helix α 5 with Thr⁶⁸⁰ in RAPTOR helix α 31. Although the GTPase domains form most of the interface with RAPTOR, there are some contacts with the CRD domains. These involve the C terminus of RagA helix α 8 (Ser²⁴³ and Lys²⁴⁴) and the N terminus of RagC helix α 8 (Gln²⁸⁰), which come close together and

engage two adjacent structural elements of RAPTOR, helix $\alpha 29$ (Thr⁶³⁹, Asn⁶⁴³, Met⁶⁴⁶) and the end of the long, mostly disordered insertion after $\alpha 31$.

Mutations of RAPTOR residues in helices $\alpha 26$ (Trp⁵⁹³-Cys⁵⁹⁴ or Arg⁵⁹⁷-Asp⁵⁹⁸) and $\alpha 29$ (Thr⁶³⁴-Asp⁶³⁵-His⁶³⁶) that contact the RagA-Q66L_{GTP} switch I/inter-switch greatly reduce binding to RagA/RagC (Fig. 3C). Previously, RagA mutations were identified that prevent RAPTOR interaction (33), and they map to the interface with RAPTOR in our structure. We also determined the cryo-EM structure of mTORC1-RagA-Q66L_{GTP}/RagC-T90N_{GDP} where RagA/RagC was not covalently fused to PRAS40. Note that in both cryo-EM structures, the RagA/RagC heterodimer interacts with RAPTOR in the same manner (figs. S7 and S8).

Cancer-associated mutations in RagA/RagC affect communication between GTPase domains

Cancer-associated mutations in RagC increase mTORC1 binding (17–19), and we wanted to

gain insights into the structural basis for this effect. The mutations cluster in various nucleotide-sensing elements of RagC: the P-loop (e.g., Ser⁷⁵), switch I (e.g., Thr⁹⁰), inter-switch (e.g., Trp¹¹⁵, Asp¹¹⁶), and switch II (e.g., Pro¹¹⁸) (fig. S1). The RagC-T90N mutant had switch regions disordered (Fig. 1D). To see whether this disorder is specific for the T90N mutation, we also determined a crystal structure of RagA-Q66L_{GTP}/RagC-S75N_{GDP} at 2.5 Å resolution (table S2). The RagC-S75N mutation in the P-loop impairs GTP binding by eliminating the interaction of Ser⁷⁵ with Mg²⁺. The structures of RagC-S75N_{GDP} and RagC-T90N_{GDP} are very similar, except that RagC-S75N_{GDP} has helix $\alpha 2$ of switch I (residues 86 to 93) ordered in one of the two heterodimers in the crystal asymmetric unit (Fig. 4A); this finding suggests that S75N destabilizes but does not completely disorder switch I. Hence, T90N causes a greater perturbation in switch I than does S75N, consistent with the more potent phenotype of

the T90N mutation in cells (17). The structure of the isolated wild-type RagC GTPase domain bound to a GTP analog [PDB ID 3LLU (34)] shows a completely ordered switch I and helix $\alpha 2$ that closely superimposes with this helix in RagC-S75N_{GDP}. In the RagC-S75N_{GDP} structure, OG1 of the Thr⁹⁰ side chain is close to the O2' of the bound nucleotide (3.7 Å), so it is likely that the larger T90N substitution leads to disorder of helix $\alpha 2$.

RagA/RagC GTPase domain contacts can be grouped into three sets (Fig. 4B). One set is at the center of the interface where the G5 motifs of the two domains meet, with RagA-Trp¹⁶⁵ near the equivalent of this residue in RagC, Tyr²²¹ (Fig. 1E and fig. S1). The second set involves interactions between RagA switch I helix $\alpha 2$ and the RagC loop immediately following the G5 motif. In particular, there is a water-mediated interaction between RagA-Arg³⁴ and the side chain of RagC-Asp²²² (Fig. 4B). The third set consists of interactions between RagC switch I helix $\alpha 2$ and the RagA

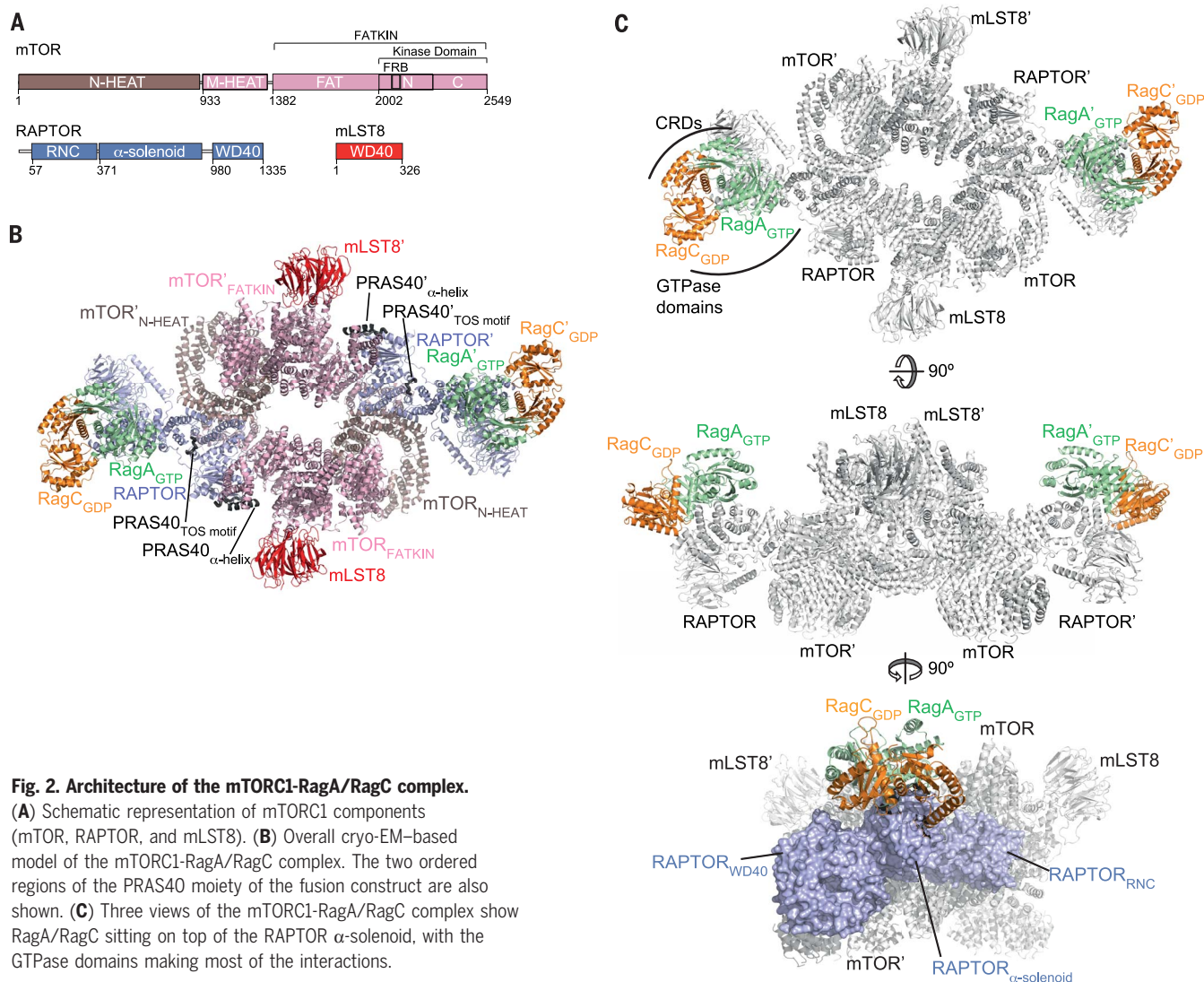


Fig. 2. Architecture of the mTORC1-RagA/RagC complex.

(A) Schematic representation of mTORC1 components (mTOR, RAPTOR, and mLST8). (B) Overall cryo-EM-based model of the mTORC1-RagA/RagC complex. The two ordered regions of the PRAS40 moiety of the fusion construct are also shown. (C) Three views of the mTORC1-RagA/RagC complex show RagA/RagC sitting on top of the RAPTOR α -solenoid, with the GTPase domains making most of the interactions.

G4/ α 5 loop. This set is present in the complex with RagC-S75N_{GDP} where RagC-Glu⁸⁹ makes a salt link with RagA-Arg¹³⁷, and RagC-Phe⁹² contacts RagA loop residues 131 to 133 (Fig. 4B), but is absent in the complex with RagC-T90N_{GDP}, where RagC switch I is completely disordered. This demonstrates that oncogenic mutations greatly perturb the interface between the GTPase domains.

Rearrangements within the RagA/RagC heterodimer when bound to mTORC1

Comparing the crystal structure of free RagA-Q66L_{GTP}/RagC-T90N_{GDP} and the cryo-EM structure of RagA/RagC bound to mTORC1 reveals a shift in the interface between the GTPase domains by ~ 7 Å (Fig. 4C and movie S1). This creates a more open space between the two GTPase domains, which in the free RagA/RagC would be kept closer by interactions involving switch I helix α 2. This might explain why the oncogenic RagC-T90N mutation, which has helix α 2 disordered, binds more easily to RAPTOR, because the RagC helix α 2/RagA interactions are already disrupted before the heterodimer binds to RAPTOR. A similar structural change could occur in the RagC-L91P mutant associated with follicular lymphomas (17). Residue Lys⁸⁴ in the α 1- α 2 loop of RagC switch I forms salt links with residues Asp²⁹⁰ and Asp²⁹⁴ of helix α 8 in the CRD (Fig. 4A). The lymphoma-associated mutation RagC-K84T (17) would likely disrupt this interaction, which could facilitate RagC rearrangement relative to RagA as seen in the complex with mTORC1.

Comparison of the CRDs from the free RagA/RagC with those in the mTORC1-bound RagA/RagC shows a small shift in the orientation of the two CRDs so that in the mTORC1-bound form, the top surface of the CRDs that embraces the GTPase domains is more splayed (fig. S9 and movie S1). Comparison of the CRD dimer from the free RagA/RagC with the CRD dimer bound to Ragulator (35) also shows shifts between the two CRDs (fig. S9). Together these results indicate that the interface between the CRD domains has some flexibility. This could be exploited by interactors such as Ragulator to exert changes on the GTPase domains via the CRDs, which could contribute to the established role of Ragulator as a GEF for RagC (27).

Structural basis for relaying nucleotide binding to the CRDs

Although only the RagA_{GTP}/RagC_{GDP} state binds RAPTOR, the reverse, inactive state, RagA_{GDP}/RagC_{GTP}, is essential for terminating mTORC1 activation. Furthermore, some Rag interactors such as galectin-8 preferentially associate with this state (36). Therefore, a structural understanding of both states is important. In the active heterodimer, nucleotide-sensitive elements in the GTPase domain of each Rag form contacts with its own CRD. In RagA_{GTP}, switch I and the β 2- β 3 interswitch firmly engage the CRD. The switch I interaction is at the center of the heterodimer interface, primarily with the CRD helix α 8, whereas the β 2- β 3 interaction is at the outer edge of the CRD (Fig. 5A and fig. S10A). These

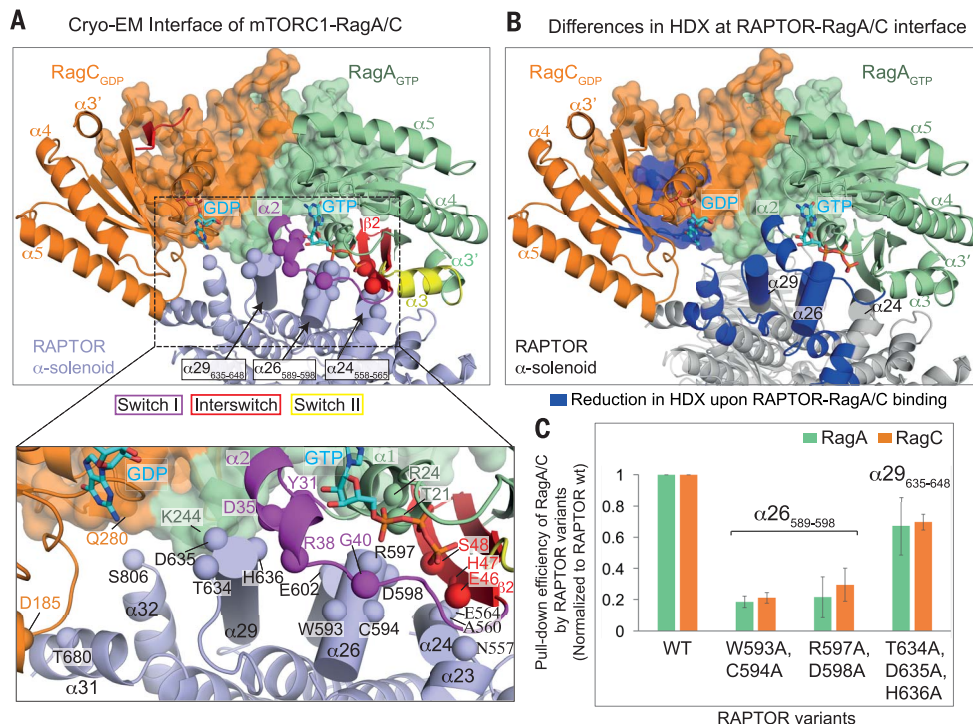
two interactions flank a central contact involving both nonswitch (α 1 and α 6) and switch (β 2- β 3) contacts with the β 7- β 8 hairpin of the CRD. In the GTP-loaded state of RagA, the tip of the interswitch protrudes beyond the GTPase domain and slips into a pocket on the surface of the CRD (Fig. 5A and fig. S10B). The interswitch tip bound in the CRD pocket may be an element of the structural basis for the “locked” state for RagA that was proposed on the basis of a kinetic study of the communication between GTPase domains in the Rag heterodimers (22). In contrast, the interswitch loop in RagC_{GDP} is in a retracted position and partially disordered. In the structure of the isolated GTPase domain of GTP-bound RagC [PDB ID 3LLU (34)], the interswitch protrudes beyond the GTPase domain in a manner equivalent to RagA_{GTP}. This is accomplished by a two-residue shift in the register of strand β 3 relative to β 1, such that residues 109 to 115 in RagC-T90N_{GDP} are in the positions of residues 111 to 117 of the GTP-bound RagC (Fig. 5B). The extended interswitch in RagC_{GTP} would clash with its CRD, which suggests that a change in the relative orientations of the GTPase and CRD domains would be required to accommodate GTP binding by RagC (fig. S10B). All of the contacts of the GTPase domain with the CRD constitute possible mechanisms for nucleotide binding to imprint onto the CRD.

Dynamics of the active and the reverse, inactive state of Rags

To gain a better understanding of the conformational changes that occur in the reverse

Fig. 3. Interface between RAPTOR and the RagA/RagC complex.

(A) Close-up views of RagA/RagC binding to the RAPTOR subunit of mTORC1. The CRDs are shown as transparent surfaces. RAPTOR helices contacting switch I and interswitch of RagA are shown as cylinders. Spheres mark RAPTOR-RagA/RagC interface residues. (B) View of the interface, illustrating regions with a decrease in HDX (blue) upon formation of the RAPTOR-RagA/RagC complex. (C) Mutational analysis of the binding interface. Strep-tagged wild-type RAPTOR (WT) and three different RAPTOR mutants (with mutations to Ala as indicated) were assayed for their ability to pull down RagA-Q66L_{GTP}/RagC-T90N_{GDP} in vitro. The pull-down efficiencies of RAPTOR mutants were normalized to WT RAPTOR. Values are means from three independent experiments; error bars denote SD.



state, we tried but did not succeed in obtaining diffracting crystals for this heterodimer. As an alternative strategy, we used HDX-MS to examine differences in conformation between the active (RagA-Q66L_{GTP}/RagC-T90N_{GDP}) and reverse (RagA-T21N_{GDP}/RagC-Q120L_{GTP}) states (Fig. 5D and table S5). Overall, both Rags showed less HDX throughout the GTPase domains when GTP-bound compared with GDP-bound, indicating a more compact domain when bound to GTP. Furthermore, upon GTP binding to RagA, there is a distinct protection in its CRD domain (e.g., in CRD helix $\alpha 8$, $\beta 7/\beta 8$ and the hinge

that are engaged with the GTPase domain), suggesting communication of the nucleotide state to the CRD (Fig. 5D and movie S2). Residues making up a pocket on the surface of the RagA CRD that accommodate the inter-switch in GTP-bound RagA have increased exchange in the reverse state, which we attribute to retraction of the RagA interswitch, exposing the RagA CRD pocket. Consistent with this, the GDP-bound RagA interswitch has increased HDX (displayed on the RagA_{GTP} crystal structure in Fig. 5D and on a RagA_{GDP} model in movie S2). For RagC, there is less change in HDX in the CRD upon GTP bind-

ing. Although the RagC interswitch shows GTP-dependent HDX protection, the pocket on the RagC CRD analogous to the RagA pocket has only small changes in protection, so currently we do not know the position of the interswitch in the GTP-bound RagC.

Implications for yeast TORC1 signaling

Comparing the yeast Gtr1_{GTP}/Gtr2_{GDP} structure [PDB ID 4ARZ (37)] with human RagA_{GTP}/RagC_{GDP} indicates very large conformational differences, both in switches and in the relative orientations of GTPase domains, with the Gtr2 GTPase domain rotated about 36° relative to the RagC GTPase domain (fig. S11A). The arrangement of the Gtr1/2 GTPase domains is not compatible with binding to mTORC1 in the same manner as RagA/RagC (fig. S11B). This suggests that the Gtr1/2 GTPase domains may reorient in order to bind Kog1, the yeast homolog of RAPTOR. The very different conformation of the Gtr2_{GDP} switch I region and the extreme orientation of the Gtr2 GTPase domain relative to RagC may reflect a fundamental difference between RagC and Gtr2. This could explain why RagA-Q66L can complement a Gtr1-deficient strain, whereas neither wild-type RagC nor a GDP-bound mutant RagC could complement Gtr2 deficiency in yeast (38). Despite these differences, the binding site of Gtrs on Kog1 maps to a similar region on the Kog1 α -solenoid (39).

Given the role of the Rag heterodimers in recruiting mTORC1 to lysosomes, the constitutive association of yeast TORC1 with the vacuole is surprising (5). A recent report elegantly showed that upon glucose starvation, yeast TORC1 forms inactive, vacuole-associated helical tubes named TORIODs and that the TOROID formation is antagonized by active Rags (Gtr1^{GTP}/Gtr2^{GDP}) in cells (40). Fitting our RagA/RagC heterodimers into the cryo-EM reconstruction of the tubes, in accordance with the arrangement present in our mTORC1-RagA/RagC complex, suggests that the Gtr1/2 binding would not be compatible with the TORC1 arrangement in the TORIODs (fig. SIIC). This might mean that Gtr1/2 binding could directly regulate assembly or disassembly of the tubes to activate TORC1. Further work is needed to test this structure-based proposal.

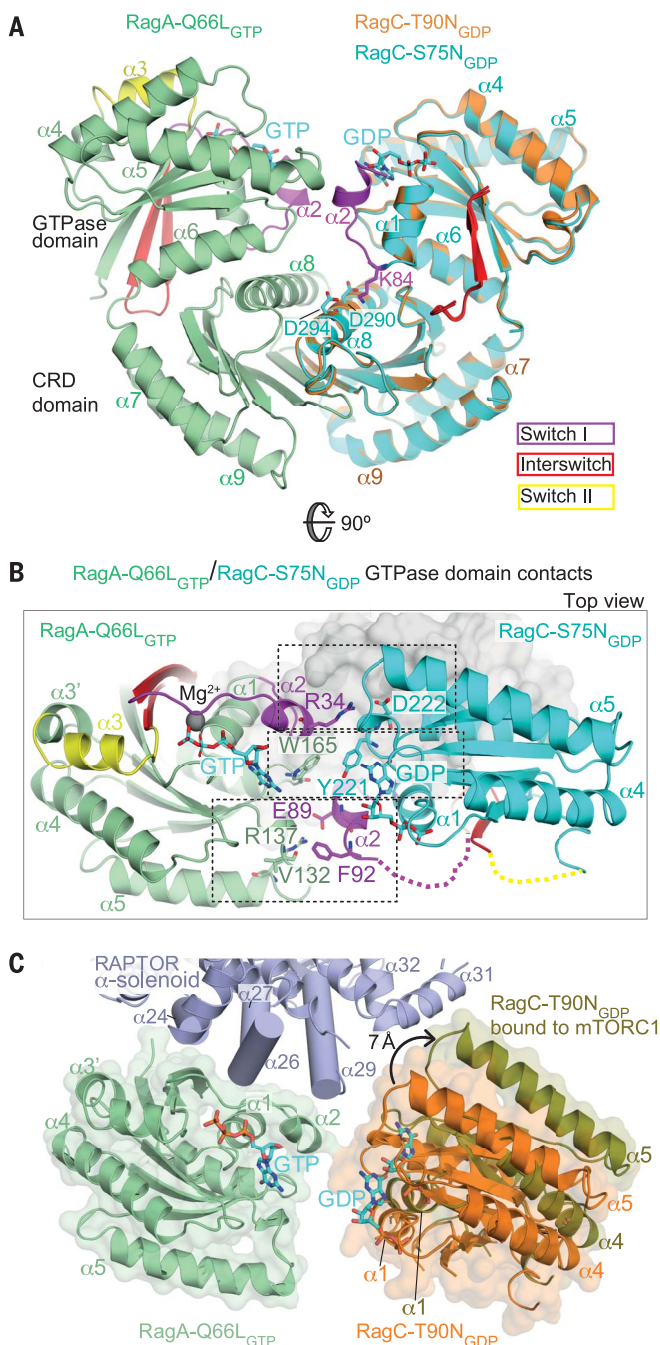
Discussion

RagA/RagC binding causes no conformational change in mTORC1, which suggests that the role of the Ragulator/Rags complex is to localize mTORC1 to lysosomes where it can be allosterically activated by RHEB. Rag/RAPTOR interaction requires a GTP-loaded RagA, so that RagA switch I and interswitch are ordered, because they make most of the interactions with RAPTOR. A reverse state of Rags with GDP-loaded RagA and GTP-loaded RagC does not bind RAPTOR as well (6, 7), because

Fig. 4. Interactions between GTPase domains in the RagA/RagC

heterodimer. (A) Comparison of RagA-Q66L_{GTP}/RagC-T90N_{GDP} with RagA-Q66L_{GTP}/RagC-S75N_{GDP}, illustrating ordering of helix $\alpha 2$ in switch I of RagC-S75N. Superposition was on the RagA subunit.

(B) Three sets of interactions between RagA_{GTP} and RagC_{GDP} GTPase domains. **(C)** A change in the orientation of RagA/RagC GTPase domains in free RagA/RagC relative to RagA/RagC bound to mTORC1. Superposition was on the RagA subunit. The view is similar to (B).



RagA_{GDP} would have the switch regions disordered, whereas RagC_{GTP} could not interact with RAPTOR, because RagC residues analogous to RAPTOR-binding residues of RagA are not conserved (fig. S12).

The structures suggest how the nucleotide-bound state of one GTPase domain is commu-

nicated both between subunits to the paired GTPase domain and within a subunit to its CRD (Figs. 4 and 5 and movie S2). First, consistent with communication between GTPase domains (22), both RagA and RagC have helix $\alpha 2$ (in switch I) contacting the paired GTPase domain and filling the space between them

(Fig. 4, A and B). Second, there are several sets of interactions between the GTPase and CRD domains within a subunit that HDX suggests are dynamic and nucleotide-dependent, including switch I and the interswitch. The interswitch of Rag GTPases apparently undergoes a nucleotide-dependent register shift of strand $\beta 3$ relative to $\beta 1$ that could be part of a mechanism to transmit nucleotide-binding information from the GTPase domain to the CRD (Fig. 5). This is analogous to conformational changes that accompany transition from the GDP- to GTP-bound states of Arf family GTPases (Fig. 5C) (41, 42) and is consistent with the evolutionary relationship of the Rags to the Arf family (21). In Arfs, this interswitch toggle between retracted and protruded conformations coordinates membrane binding with GTP loading (Fig. 5B). In Rags, the interswitch toggle could be part of a mechanism that rotates one GTPase domain via a CRD fulcrum relative to the other GTPase domain. This would change RagA/RagC GTPase domain contacts, making it less favorable for the heterodimer to accommodate GTP in both GTPase domains at the same time, as kinetically observed (22).

mTORC1 activity is intricately regulated in a signal- and location-specific manner. Membrane compartments act as signaling platforms that serve to colocalize mTORC1 with its activating G protein RHEB, which is targeted transiently to most endomembranes by farnesylation (13). The lysosomal activity of mTORC1 in amino acid signaling is achieved through its dynamic interface with the Rags-Ragulator lysosomal scaffold (8, 43). Rags couple mTORC1 to lysosomes by binding to RAPTOR with their GTPase domains and to Ragulator with their CRDs. Because of large allosteric changes in mTOR that are incompatible with a mixed mTOR dimer, two RHEB molecules bind mTORC1 cooperatively (3). In contrast, two soluble RagA/RagC heterodimers bind independently to mTORC1, because RagA/RagC binding does not introduce conformational changes in mTORC1.

We propose an organization of active mTORC1 on membranes based on our structure of the mTORC1-RagA/RagC complex, the previously published structure of the mTORC1-RHEB complex (3), and the crystal structure of Ragulator bound to RagA/RagC CRDs (35, 44) (Fig. 6). In this model, RagA/RagC, associated with membranes through Ragulator (via the lipidated N terminus of LAMTOR1), and RHEB, associated with membranes through C-terminal farnesylation, can be bound at the same time to mTORC1, yet still allow the mTOR active sites to face the cytosol. The RHEB-binding surface of mTORC1 would be near a RHEB-containing membrane, and the first ordered residue of the LAMTOR1

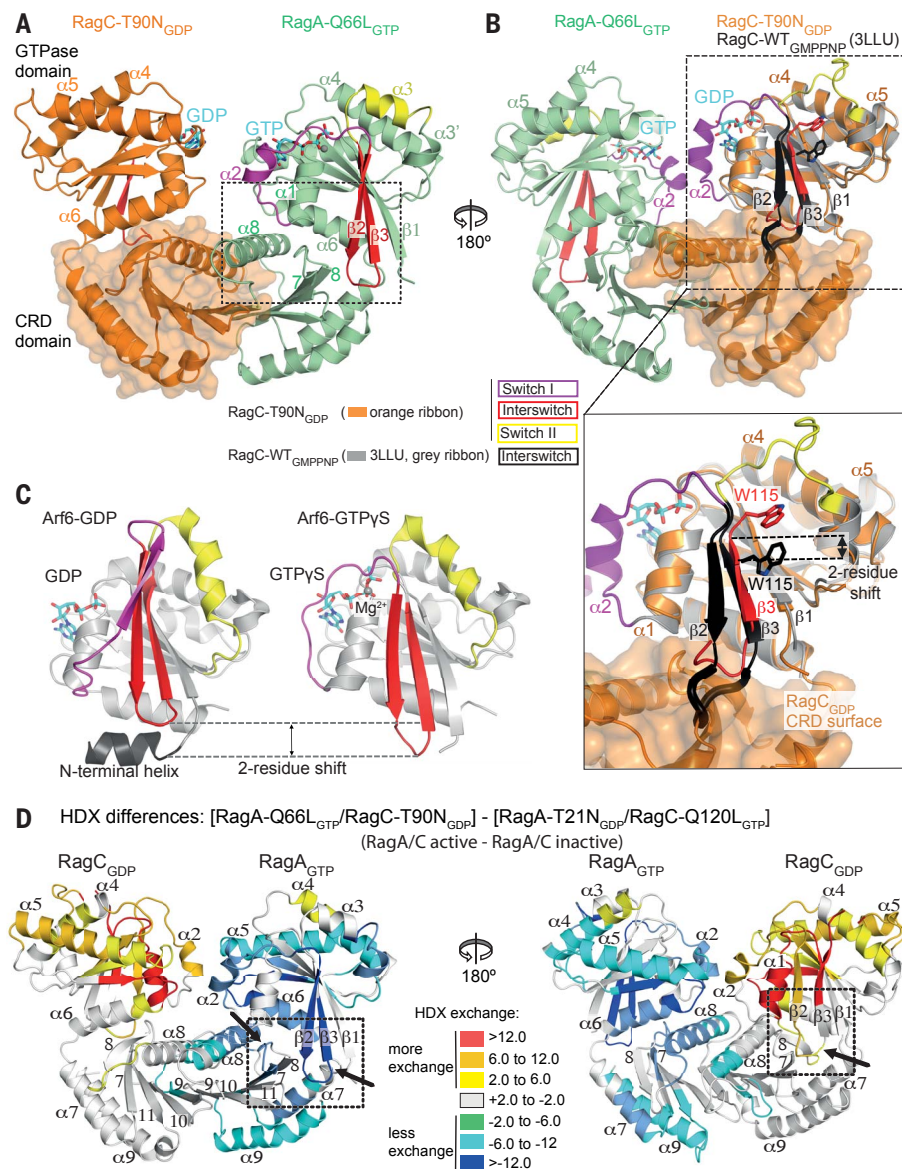


Fig. 5. Structural basis for communicating nucleotide binding within the RagA/RagC heterodimer.

(A) Both switch I and the interswitch make nucleotide state-dependent direct contacts with the CRD. (B) Superposition of GTP-bound RagC (PDB ID 3LLU, in grey) on the GDP-bound RagC (from our RagA-Q66L_{GTP}/RagC-T90N_{GDP} complex). The interswitch of GTP-bound RagC is black; GDP-bound RagC is red. The Trp¹¹⁵ position illustrates a two-residue shift in strand $\beta 3$ (relative to strand $\beta 1$). The $\beta 2/\beta 3$ loop toggles between a retracted conformation in the GDP state and an extended conformation in the GTP state that would clash with the CRD if there were no conformational changes. (C) The structures of Arf6 bound to either GDP [PDB ID 1E0S (45)] or GTP γ S [PDB ID 2J5X (46)], with switches colored as in (A). The interswitch toggle couples nucleotide binding with membrane binding by the N-terminal helix. (D) Differences in HDX between the active (RagA_{GTP}/RagC_{GDP}) and inactive (RagA_{GDP}/RagC_{GTP}) states, illustrating changes in the CRDs in addition to the expected changes in the GTPase domains. In RagC_{GDP}, disordered regions in the switches have been modeled to illustrate all of the HDX changes.

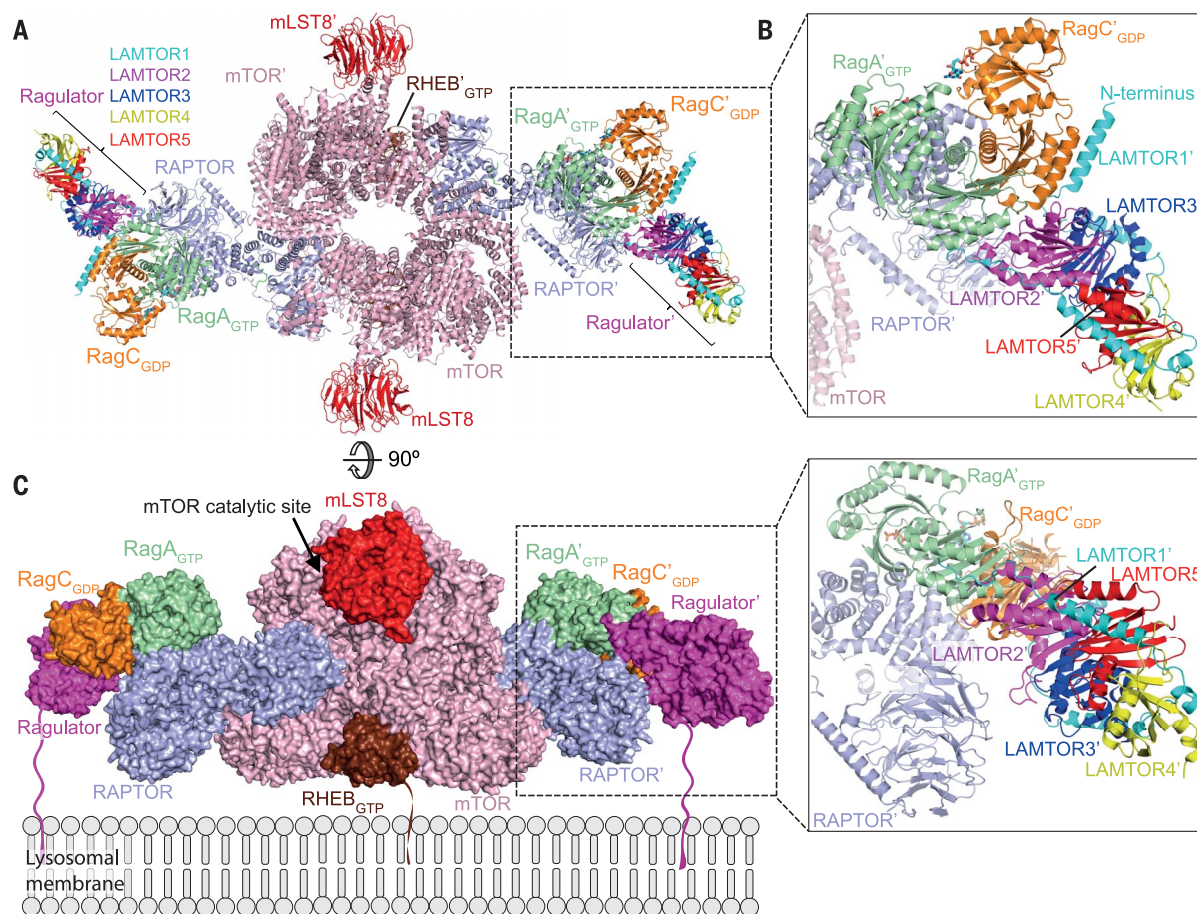


Fig. 6. Model of the mTORC1-RHEB-RagA/RagC-Ragulator complex. (A) A ribbon diagram of the mTORC1-RHEB-RagA/RagC-Ragulator complex. RAPTOR-RagA/RagC was superimposed on RAPTOR in mTORC1-RHEB complex [PDB ID 6BCU (3)]. The CRD of the mTORC1-RagA/RagC-RHEB complex was superimposed onto the

CRD of the crystal structure of the Ragulator-CRD domain complex [PDB ID 6EHR (35)]. (B) Expanded view of the Ragulator/Rags/RAPTOR interface. (C) A model of the complex on a lysosomal membrane. The lipid-modified regions of LAMTOR1 and RHEB that anchor them to membranes are depicted in arbitrary conformations.

subunit of Ragulator (residue 96) would be about 105 Å from the membrane surface, which suggests that the 95 flexible N-terminal LAMTOR1 residues could easily reach the membrane. Further structural and kinetic analysis of mTORC1 complexes on membranes will be essential to fully appreciate the roles of structural dynamics of mTORC1 with its regulators and the roles of membranes in regulation of mTORC1.

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analyzed data. M.A., O.P., D.M.S., and R.L.W. developed the experimental plan. M.A. and R.L.W. wrote the draft. All authors reviewed and edited the drafts. **Competing interests:** Authors declare no competing interests. **Data and materials availability:** Materials supporting findings are available from R.L.W. upon reasonable request. Coordinates for crystal structures of RagA/RagC GTPase heterodimers were deposited in the Protein Data Bank, accession codes 6S6A and 6S6D. The cryo-EM reconstructions of the mTORC1-RagA/RagC complex was deposited with EMDB (EMD-10132 and EMD-10133) and PDB (entries 6S60 and 6S62).

SUPPLEMENTARY MATERIALS

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PHASE-CHANGE MEMORY

Phase-change heterostructure enables ultralow noise and drift for memory operation

Keyuan Ding^{1,2*}, Jiangjing Wang^{3,4*}, Yuxing Zhou^{3*}, He Tian^{5*}, Lu Lu⁶, Riccardo Mazzarello⁷, Chunlin Jia^{6,8}, Wei Zhang^{3†}, Feng Rao^{1,9†}, Evan Ma^{10†}

Artificial intelligence and other data-intensive applications have escalated the demand for data storage and processing. New computing devices, such as phase-change random access memory (PCRAM)-based neuro-inspired devices, are promising options for breaking the von Neumann barrier by unifying storage with computing in memory cells. However, current PCRAM devices have considerable noise and drift in electrical resistance that erodes the precision and consistency of these devices. We designed a phase-change heterostructure (PCH) that consists of alternately stacked phase-change and confinement nanolayers to suppress the noise and drift, allowing reliable iterative RESET and cumulative SET operations for high-performance neuro-inspired computing. Our PCH architecture is amenable to industrial production as an intrinsic materials solution, without complex manufacturing procedure or much increased fabrication cost.

The rapid development of artificial intelligence (AI), big data analytics, and supercomputing demands a fundamental change in the current computing systems based on von Neumann architecture (1–7), which is characterized by physically separated processing and storage units. Their intercommunication across bandwidth-limited and energy-inefficient interconnects constitutes a bottleneck for data shuffling, leading to a 40% power waste. Chalcogenide phase-change materials (PCMs)-based random-access memory (PCRAM) (7–14), available as storage-class memory in the memory market (15), can mitigate to some extent the performance mismatch between dynamic random access memory (DRAM) and flash-memory-based solid-state drives. But to fundamentally break the aforementioned bottleneck, non-von Neumann computing architecture that unifies

computing with storage in memory cells, such as neuro-inspired computing (1–7), needs to be pursued. Resistive memories such as PCRAM are a promising route for the implementation of this new form of computing (2–7). This, however, sets a high bar for the performance of PCRAM.

PCRAM products currently use $\text{Ge}_2\text{Sb}_2\text{Te}_5$ (GST) as the core material for memory programming. Digital information is encoded through reversible transformation between the amorphous and crystalline phases of GST. The unconstrained three-dimensional phase transitions, crystallization (SET operation), and melt-quenched amorphization (RESET operation) cause composition deviations and structural variations upon extensive cycling. This results in large fluctuations in electrical resistance of both the SET (crystalline) and RESET (amorphous) states (16). Structural relaxation also takes place in amorphous GST, resulting in the steady increase in electrical resistance with time at ambient temperatures that is known as the resistance drift (17). For binary storage, these noise and drift issues are not critical because the resistance contrast between amorphous and crystalline GST is greater than two orders of magnitude and clearly distinguishable at all times. However, the strong intra- and intercell variability becomes a formidable challenge when it comes to the implementation of neuro-inspired computing as in-memory computing (18–21), deep neural networks (22), and spiking neural networks (23–25).

Recent device advances to address this challenge—which include the mixed-precision in-memory computing scheme (18), the projected phase-change memory scheme (26, 27), and the multi-PCRAM cells scheme (22, 23)—have resulted in much improvement in the speed-up and energy-saving factors for key computing tasks, such as vector-matrix multiplications (18), pattern recognitions (22), temporal correlation detection (28), and other machine-

learning tasks (29). However, these remedies (i) mostly improve the RESET aspect and cannot resolve the randomness issue associated with the SET operations for machine-learning tasks that require high accuracy and consistency, (ii) complicate the chip-system architecture and the inherent working mode, hindering its integration into mass-produced chips, and (iii) are largely extrinsic help from the device setup and connection standpoint. Therefore, an intrinsic solution that improves each and every PCRAM cell is a pressing need (30). One solution is an innovative materials design that enables low noise, small drift, long cycling endurance, fast switching, and high energy efficiency. We propose and have demonstrated a phase-change heterostructure (PCH) that simultaneously resolves all of these critical issues, opening a different avenue toward high-performance phase-change computing chips.

Design principles and fabrication of a PCH device

For typical T-shaped phase-change devices fabricated by use of the 0.13- μm node complementary metal-oxide semiconductor (CMOS) technology, the programming (amorphous-crystalline phase transformation) region in the PCM layer forms a mushroom-like shape (Fig. 1A). To avoid the long-distance atomic transport along the pulsing direction, the conventional monolithic PCM needs to be subdivided into compartments. We therefore replaced the active layer with a multilayer PCH composed of alternately deposited nanolayers of a confinement material (CM) and a PCM (Fig. 1B). The materials selection was based on the following design principles, leading to six criteria that should be met simultaneously for superior device performances. The performance includes not only low variability and drift but also long cycling endurance, low power consumption, and high switching speed.

First, we required the CM layers to be dynamically robust in dense grids, acting as effective diffusion barriers against long-range atomic transport along the pulsing direction upon extensive cycling. The CM crystal should therefore have a much higher melting temperature (T_m) and smaller in-plane lattice parameter than that of the PCM layer. Second, the CM should be chemically unreactive and weakly coupled (for example, through weak covalent and/or van der Waals interactions) with the PCM layer, to keep the heterostructure interfaces intact at all operation temperatures. Strong CM-PCM interfacial bonding needs to be avoided because it may pin down the outer atomic layers in the PCM slab toward its crystalline structure, making the remaining amorphous fraction difficult to be sustained at nanoscale (31, 32). Third, the CM layers should have local (short to medium range) atomic arrangements (such as octahedral motifs) similar

¹College of Materials Science and Engineering, Shenzhen University, Shenzhen 518060, China. ²Key Laboratory of Optoelectronic Devices and Systems of Ministry of Education and Guangdong Province, College of Optoelectronic Engineering, Shenzhen University, Shenzhen 518060, China.

³Center for Advancing Materials Performance from the Nanoscale, State Key Laboratory for Mechanical Behavior of Materials, Xi'an Jiaotong University, Xi'an 710049, China.

⁴School of Chemistry and Chemical Engineering, Yulin University, Yulin 719000, China. ⁵Center of Electron Microscopy, State Key Laboratory of Silicon Materials, School of Materials Science and Engineering, Zhejiang University, Hangzhou 310027, China. ⁶The School of Microelectronics, State Key Laboratory for Mechanical Behavior of Materials, Xi'an Jiaotong University, Xi'an 710049, China. ⁷Institute for Theoretical Solid State Physics, JARA-FIT and JARA-HPC, RWTH Aachen University, Aachen 52074, Germany. ⁸Ernst Ruska-Centre for Microscopy and Spectroscopy with Electrons, Forschungszentrum Jülich GmbH, Jülich 52425, Germany. ⁹State Key Laboratory of Functional Materials for Informatics, Shanghai Institute of Micro-system and Information Technology, Chinese Academy of Sciences, Shanghai 200050, China. ¹⁰Department of Materials Science and Engineering, Johns Hopkins University, Baltimore, MD 21218, USA.

*These authors contributed equally to this work. [†]Corresponding author. E-mail: wzhang0@mail.xjtu.edu.cn (W.Z.); fengrao@szu.edu.cn (F.R.); ema@jhu.edu (E.M.).

to that of the crystalline PCM counterpart, which could help speed up crystallization of the amorphous PCM. Fourth, the CM layers need to be thermally resistive to serve as effective barriers to prevent through-plane heat loss during programming, thus reducing the required programming bias (energy). Fifth, the CM layers should have similar electrical resistivity as compared with that of the PCM layers, maintaining the resistance window suitable for programming. Sixth, the CM selection should be compatible with the current PCMs and CMOS technology, without involving undesirable or excessive elements.

After we screened potential candidate systems on the basis of the above criteria, we found that the transition-metal dichalcogenides MX_2 —with $\text{X} = \text{Te}$ and $\text{M} = \text{Ti}, \text{Zr}, \text{Ni}$, or Mo , for example—were suitable choices for the CM. Although there are plenty of MX_2 -PCM combinations, we used $\text{CM} = \text{TiTe}_2$ and $\text{PCM} = \text{Sb}_2\text{Te}_3$ because this combination was experimentally verified to be weakly coupled (33). The $\text{TiTe}_2/\text{Sb}_2\text{Te}_3$ couple satisfies all our criteria because TiTe_2 has a much higher T_m of ~ 1470 K, a lower thermal conductivity of $\sim 0.12 \text{ W m}^{-1} \text{ K}^{-1}$, a comparable electrical resistivity of $\sim 1.3 \times 10^{-6} \text{ ohm m}$, and a similar octahedral arrangement with a smaller in-plane lattice parameter of $\sim 3.77 \text{ \AA}$ (Fig. 1C) in comparison with Sb_2Te_3 (~ 900 K, $\sim 0.78 \text{ W m}^{-1} \text{ K}^{-1}$, $\sim 3 \times 10^{-5} \text{ ohm m}$, and $\sim 4.26 \text{ \AA}$, respectively) (Fig. 1D) (33). Density functional theory (DFT) calculations (34) indicate a more favorable cohesive energy of TiTe_2 (-0.694 eV per atom) as compared with Sb_2Te_3 (-0.119 eV per atom). Crystal orbital Hamilton population (COHP) analyses (34) indicate that both TiTe_2 (Fig. 1C) and Sb_2Te_3 (Fig. 1D) are chemically robust, as evidenced by the absence of antibonding interaction at the Fermi level. In particular, the Ti-Te bonds are of high chemical strength owing to the pure stabilizing contribution below the Fermi level.

We fabricated the T-shaped $\text{TiTe}_2/\text{Sb}_2\text{Te}_3$ PCH device using the $0.13\text{-}\mu\text{m}$ node CMOS technology with a tungsten bottom electrode contact (BEC) ~ 190 nm in diameter (Φ) (34). We deposited the PCH film, ~ 69 nm in total thickness, at $\sim 300^\circ\text{C}$ (substrate temperature) by sputtering TiTe_2 and Sb_2Te_3 targets alternately. The thicknesses of each TiTe_2 and Sb_2Te_3 sublayer are ~ 3 and ~ 5 nm, respectively. The in situ heating during deposition was necessary for growing high-quality heterostructures because they would otherwise show conventional alloy-like behavior with strong fluctuation and drift in electrical resistance (35). The initial direct current-voltage sweep of the pristine (as-fabricated) PCH device exhibited a highly conductive linear ohmic relationship (fig. S1) because all the PCM and CM nanolayers were fully crystallized. For subsequent operations, we observed the threshold-switching behavior (11), indicating that the Sb_2Te_3 sublayers were in the amorphous state after each RESET oper-

ation (fig. S1). We prepared a GST device with the same geometry for comparison.

Ultralow noise for memory programming

We systematically characterized electrical and structural properties of the PCH devices and PCH thin films. We suppressed the noise of the PCH device: The fluctuations in electrical resistance of both RESET (Fig. 2A) and SET (Fig. 2B) states were strongly reduced compared with those of the GST device. The relative standard deviations of RESET and SET states of the PCH are 1.8 and 0.4%, respectively, which are about one order of magnitude lower than those of the GST device (12.4 and 7.2%, respectively). In conventional PCRAM devices, PCMs are subjected to nonisothermal and nonequilibrium conditions, which result in severe composition variation upon extensive programming owing to long-range diffusional redistribution of constituent elements along the electrical current direction (11). Eventually, phase segregation occurs, rendering the device unreliable. In our PCH device, the TiTe_2 sublayers with sustained integrity divided the switching component into multiple ~ 5 -nm slabs along the pulsing direction, reducing the possibility and extent of compositional variation and phase segregation. During the quasi-two-dimensional switching in these compartmentalized Sb_2Te_3

nanolayers, the randomness of phase transitions (the stochastic crystallization in particular) was markedly reduced, leading to more consistent resistance contrast and hence better-defined logic states. The simplified switching component by itself, from ternary GST to binary Sb_2Te_3 , can contribute to the reduction of stochasticity in crystallization (9); however, Sb_2Te_3 alone without TiTe_2 multilayers is known to show large noise and poor cycling endurance (36).

We used DFT-based molecular dynamics (DFMD) simulations (34) to validate from the modeling side the highly confined quasi-two-dimensional phase change. We constructed a periodically repeated $\text{TiTe}_2/\text{Sb}_2\text{Te}_3$ PCH supercell and heated it up to 1300 K. At this high temperature, the Sb_2Te_3 slab quickly melted, but the TiTe_2 slab remained intact (fig. S2). The atomic diffusion coefficient profile at 1300 K along the vertical direction, D_v , in the center of the Sb_2Te_3 slab was comparable with that of bulk liquid Sb_2Te_3 but decreased rapidly to zero when approaching the TiTe_2 slab (Fig. 2C). We did not observe a single Sb or Te atom penetrating the dense grids formed by TiTe_2 during the DFMD simulations at 1300 K for more than ~ 100 ps. Liquid Sb and Te atoms diffused close to the boundaries, but all bounced back (Fig. 2C). In addition, the spatial gap and the absence of stable bonds formed between

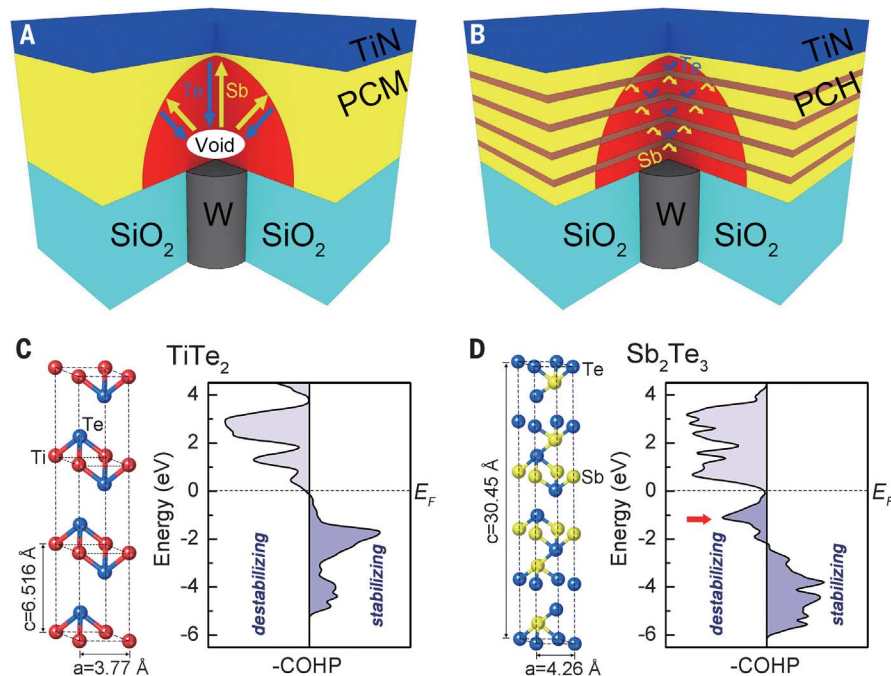


Fig. 1. Material design. (A and B) Sketch showing the switching of PCM and PCH in mushroom-type PCRAM devices. Yellow areas represent the crystalline state of PCMs, and orange slabs indicate the confinement layers. The red region is the effective programming area in contact with crystalline surroundings, and the arrows denote atomic diffusion occurring in the liquid and supercooled liquid states. The diffusion directions are along that of the input electrical pulse with a given bias polarity. For PCMs, a void could form near BEC upon extensive cycling. (C and D) The atomic structure and COHP analyses of TiTe_2 and Sb_2Te_3 crystals. The left and right parts of the -COHP curve indicate antibonding (destabilizing) and bonding (stabilizing) interactions, respectively. The red arrow marks an antibonding region in Sb_2Te_3 right below its Fermi level E_F .

the Sb_2Te_3 and the TiTe_2 slabs confirmed that they are weakly coupled. At the lower temperatures used for programming (500 to 900 K), atomic diffusion across these interslab boundaries is even more unlikely because the kinetic energy of atoms is further reduced.

Using transmission electron microscopy (TEM), we directly observed the preferential amorphization of the PCM layer in the PCH architecture (34). Amorphization can be achieved in GST thin films under irradiation of strong electron beams with high accelerating voltage and high beam intensity (37). This amorphization route circumvents the evaporation issue that prevents direct observations of melt-quench amorphization in unencapsulated thin films inside TEM. We performed similar in situ irradiation experiments on the PCH thin films on a FEI Titan G2 transmission electron microscope. Many local areas in Sb_2Te_3 slabs turned amorphous after 5 min irradiation, whereas all TiTe_2 crystalline slabs remained unchanged (Fig. 2D and fig. S3). The fast Fourier transform (FFT) analyses of typical areas in Sb_2Te_3 slabs

(for example, Fig. 2D, boxed regions) showed a clear difference. The vertical stripe-spot pattern for the crystalline phase was in sharp contrast with the halo pattern for the amorphous phase (Fig. 2D). We conducted additional chemical mapping of the PCH thin film (fig. S3) to support the quasi-two-dimensional switching mode.

Ultralow drift in RESET state

The RESET state (obtained with 2.15 V pulses over 20 ns) of the $\text{TiTe}_2/\text{Sb}_2\text{Te}_3$ PCH device (Fig. 3A, inset) showed a very low-resistance drift coefficient (v) of only ~ 0.002 at room temperature (Fig. 3A, black curve), which is comparable with its SET state (Fig. 3A, red curve). Repeated tests of the RESET states showed consistently low drift of $v < 0.005$. This is in stark contrast with the GST device in its fully amorphous state, where $v \sim 0.11$ (17) is more than 50 times higher.

To confirm that the low drift indeed corresponds to the nanoscale amorphous PCM layers, we monitored the cell resistance drift behavior of a T-shaped PCRAM device fabricated based

on $\sim 150\text{-nm}$ pure Sb_2Te_3 in contact with a $\sim 190\text{-nm}$ -diameter W BEC (Fig. 3B, inset) at room temperature. The RESET state of this device had a drift coefficient of $v \sim 0.066$ (Fig. 3B), which is about 60% of the GST device of similar size $v \sim 0.11$ (17). We scaled down the thickness of the Sb_2Te_3 thin film to $\sim 5\text{ nm}$ and measured its sheet resistance at room temperature. T-shaped PCRAM devices with $\sim 5\text{ nm}$ Sb_2Te_3 were not considered because of the challenge in fabricating and reliably programming (31). The sheet resistance of the $\sim 5\text{-nm}$ Sb_2Te_3 thin film, sandwiched by highly resistive SiO_2 layers (Fig. 3C, inset), also showed a very small drift coefficient of $v \sim 0.005$ (Fig. 3C) at room temperature. We further verified using TEM experiments that the $\sim 5\text{-nm}$ Sb_2Te_3 thin film was fully amorphous because we observed no obvious crystallites in the high-resolution TEM image, and the corresponding electron diffraction pattern showed dim halos (Fig. 3D). The electrical response of the $\sim 5\text{-nm}$ amorphous Sb_2Te_3 thin film compares well with the RESET state of $\sim 69\text{-nm}$ PCH device

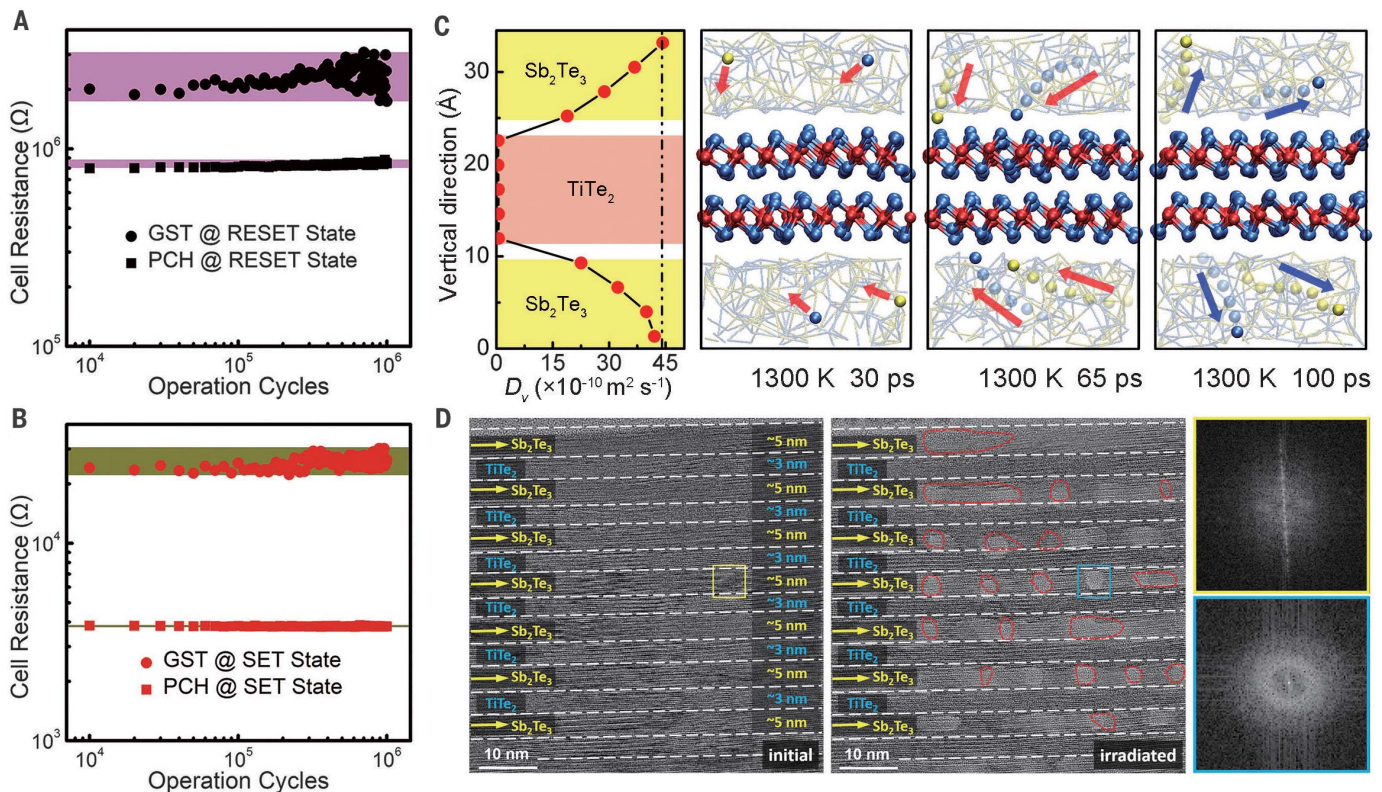


Fig. 2. Prohibited elemental diffusion. (A and B) Stable resistance values are found for RESET and SET states of the PCH device consistently, in stark contrast with the GST device. The programming was carried out with 10-ns voltage pulses of 2.3 and 1.7 V to RESET and SET, respectively, the PCH device and 50-ns voltage pulses of 5.0 and 2.5 V to RESET and SET, respectively, the GST device. (C) The profile of the diffusion coefficient of the heterostructured model along the vertical direction extracted from the DFMD simulations at 1300 K. The dashed line marks the diffusion coefficient of bulk Sb_2Te_3 . The diffusion paths of some liquid-like Sb and Te atoms are highlighted. These simulations are subjected to isothermal conditions;

therefore, Sb and Te atoms diffuse in random directions, which is different from the situation in devices, in which Sb and Te atoms diffuse oppositely along the pulsing direction. Ti, Sb, and Te atoms are rendered as red, yellow, and blue spheres, respectively. Most of the atoms in the Sb_2Te_3 are indicated by using the chemical bonds formed between them, whereas sticks and balls are used for crystalline TiTe_2 . (D) Under irradiation with strong electron beams for 5 min, parts of the Sb_2Te_3 sublayers of a PCH thin film turn amorphous, whereas the TiTe_2 sublayers remain entirely crystalline. A typical area in one Sb_2Te_3 sublayer is analyzed by using FFT, which clearly tells apart the crystalline and amorphous phases.

having multiple ~5-nm Sb_2Te_3 sublayers, but not with the 150-nm-thick Sb_2Te_3 device.

We found two reasons for the low drift in our PCH device. First, the PCH presents simplified chemical bonding and structural motifs in amorphous Sb_2Te_3 compared with closely related PCMs such as GeTe and GST. In the amorphous state of GeTe and GST, the resistance drift is mainly caused by the diminishing content of structural defects such as tetrahedral Ge motifs and the reinforcement of Peierls distortion of (defective-) octahedral motifs (38). In amorphous Sb_2Te_3 , both Ge and tetrahedral defects are no longer present, hence eliminating one of the driving forces for structural relaxation. This scenario holds for amorphous Sb_2Te_3 in both bulk (39) and PCH, as confirmed with our DFMD calculations. Second, the PCH restricts structural relaxation because of nanosize effects. Nanoscale walls were reported to slow down the dynamics of supercooled metallic liquids, restricting the structural relaxation near the walls (40). Similarly, the atomic diffusion dynamics of supercooled Sb_2Te_3 liquids decayed rapidly when approaching the TiTe_2 walls

(Fig. 2C and fig. S4A). The Te-Te antibonding interaction in between the wall and the nearby Te-Sb-Te bonding pairs of Sb_2Te_3 hindered the reinforcement of Peierls distortion toward the walls (fig. S4B). Visible gaps were consistently found between these two sublayers upon quenching the PCH model down to 0 K (fig. S5).

High-accuracy iterative RESET and cumulative SET operations

The key property of PCRAM that enables neuro-inspired computing is its ability to achieve a continuum of electrical resistance values, not just the two basic levels discussed above. The continuous change in electrical resistance can be achieved by either the iterative RESET operation or the cumulative SET operation. Instead of sending a strong voltage pulse for accomplishing a complete RESET operation (Fig. 3A), we applied the RESET pulses with progressively increased amplitude (Fig. 3E, inset). The pulsing scheme resulted in an iterative RESET process with increasing volume of programming region and allowed for multiple intermediate

resistance states (Fig. 3E) because the memory cell has a varied crystalline/amorphous ratio (11). The GST devices, however, suffered from serious resistance drift (17), in which the adjacent resistance states with close magnitude would be difficult to resolve and may even overlap after a certain period of time, leading to decoding errors (11). By contrast, we managed to achieve seven intermediate states with consistently low drift coefficients ($v \leq 0.005$) over 1 hour (Fig. 3E) by setting a fixed pulse width of 20 ns and varied pulse amplitude from 1.95 to 2.15 V in our PCH device. We performed the same iterative RESET operation over four additional PCH cells and obtained consistent results with small relative standard deviations below 5.5% (fig. S6). This device property resolves the imprecision and inconsistency issue of PCRAM cells, owing to the suppressed structural relaxation of amorphous Sb_2Te_3 sublayers at room temperature. These 9-level precision memory cells are already well suited for high-accuracy vector-matrix multiplications and pattern classifications (18, 27), and further tuning of the programming parameter and the PCH

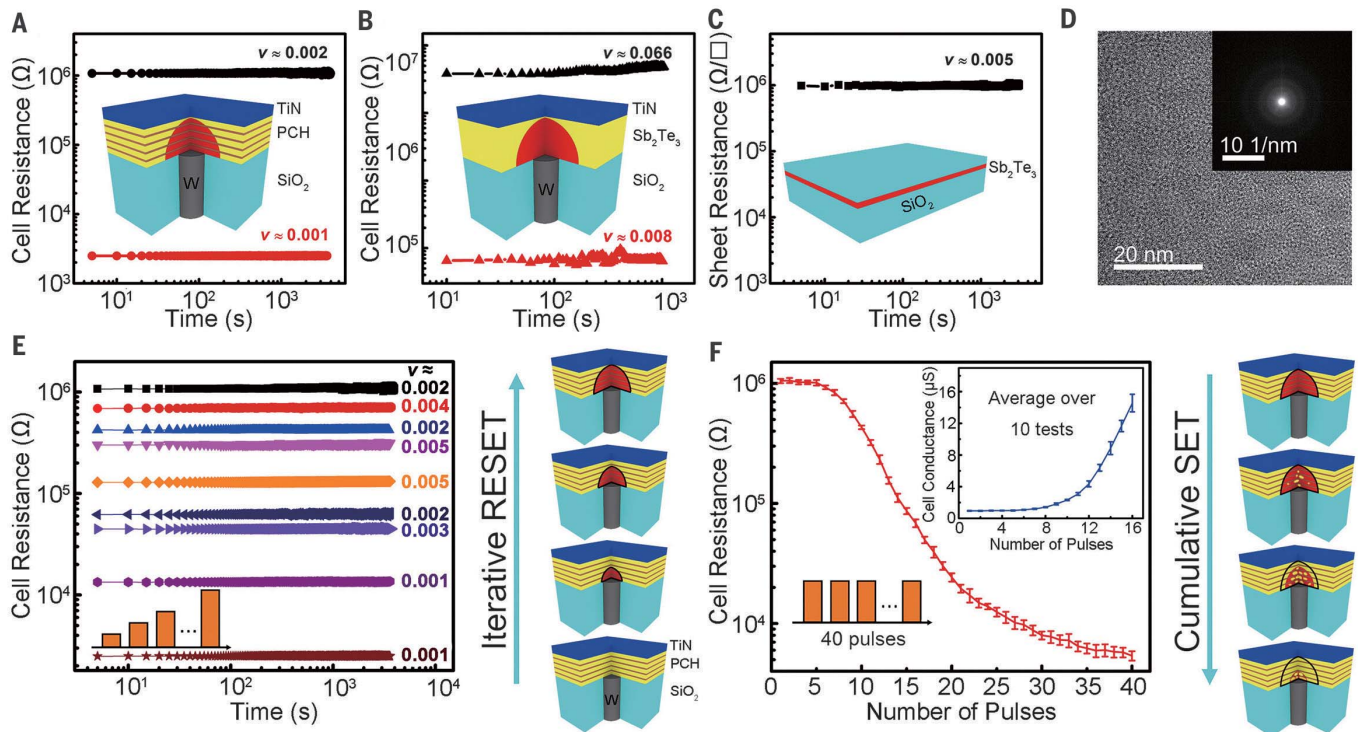


Fig. 3. Suppressed resistance drift. (A) Cell resistance as a function of time for the RESET and SET state of the PCH device. (B) Cell resistance as a function of time for the RESET and SET state of a ~150-nm-thick Sb_2Te_3 device, with the underlying W plug of ~190 nm in diameter. (C) Sheet resistance as a function of time for a ~5-nm-thick amorphous Sb_2Te_3 thin film sandwiched between SiO_2 layers. (Insets) The sketch of the device setup in (A) and (B), and the thin film configuration in (C). All the curves were measured at room temperature. The change of resistance obeys the power law, $R(t) = R_0 (t/t_0)^v$, where R_0 is the initial resistance at t_0 , and v is the fitted resistance drift coefficient. (D) The high-resolution TEM image and corresponding electron diffraction pattern of the ~5-

nm Sb_2Te_3 film, confirming the film to be in a fully amorphous state.

(E and F) Iterative RESET and cumulative SET operations. (Insets) The pulse waveforms. The sketch plots show the size of programming areas (black lines) and the change in effective amorphous volumes (red areas). The iterative RESET operation was done by setting a fixed pulse width of 20 ns and varied pulse amplitude from 1.95 to 2.15 V. The cumulative SET operation was done by sending a train of 40 voltage pulses with the same magnitude (0.8 V) and the same width (100 ns). The same operation was repeated 10 times. The top inset in (F) shows the computed conductance (with standard deviation) converted from the resistance data.

geometry should be able to readily increase the number of reliable intermediate states (fig. S7).

Higher-level in-memory (such as machine-learning) tasks and integration of neural networks with and without emulations of synapses and neurons are in general demanding, in terms of maximum tolerable fluctuations, on cumulative SET operation (29). A cumulative SET operation is done by sending a train of narrow voltage pulses with the same low amplitude (Fig. 3F, inset), corresponding to programming areas of the same size (Fig. 3F, inside the black lines). Unlike iterative RESET, in which the melt-quench amorphization is abrupt and the resistance of multilevel states changes as a function of the amorphous/crystalline ratio, the cumulative SET operation is accomplished through incubation of crystal nuclei, their subsequent growth, and parallel mushroom boundary shrinkage, leading to a nonlinear reduction in cell resistance (Fig. 3F).

We performed the cumulative SET operation using a train of 40 identical voltage pulses (0.8 V and 100 ns of each pulse) on the same PCH cell 10 times. As designed, the multiple TiTe_2 walls prevent the formation of polycrystalline grains vertically because the typical grain size of nucleation-type PCMs is 5 to 20 nm. In addition, similar local atomic motifs in these walls may promote crystallization of Sb_2Te_3 sublayers through potential interface nucleation. As expected, consistent resistance (with $\leq 10\%$ fluctuation) is found at each and every point along the path from the RESET state to the SET state (covering the full resistance contrast window of more than 2 orders of magnitude), for the 10 tests of the same PCH device (Fig. 3F and fig. S8). We also computed the conductance, because neural network emulations use this variable, and set a similar window below $15 \mu\text{S}$ (Fig. 3F, top inset) to compare with the GST devices of similar configuration (29). The relative standard deviation in conductance is

below 9% for a single PCH device, in contrast with the GST device ($>40\%$) (29). We carried out the same cumulative SET operation over four additional PCH cells, and the intercell fluctuation in conductance was below 11% (fig. S9). We attribute the highly repeatable conductance of the PCH device to the reduced structural variability of crystallization (including the size, volume fraction, and crystallographic orientation of the crystallized grains) and the decreased pulse-to-pulse composition variation, made possible by the robust confinement layers. Advances in this direction are highly promising for many machine-learning tasks, such as unsupervised learning of temporal correlations between binary stochastic processes (28). Further reduction in the randomness associated with crystallization may be possible by tuning the thickness and stoichiometry (9) of the PCM nanolayers.

Improved programming energy, speed, and cycling endurance

Our PCH architecture leads to much improved RESET energy, SET speed, and cycling endurance compared with those of conventional GST devices. The required voltage bias for RESET operations (Fig. 4A) in the PCH device is ~ 2.0 to 2.5 V when using sub-10-ns voltage pulses, and much lower than that for the GST device, ~ 3.5 to 6.1 V when using several tens of nanosecond voltage pulses, meeting the low-bias criterion for mass-produced chips (41). To make an accurate estimate of RESET energies, we used a very wide constant electric current pulse of 1000 ns (t). The RESET energy (E) is then calculated as $E = I \times U \times t$, resulting in 0.91 and 7.35 nJ for the PCH and GST device, respectively (where I is the RESET current and U is the RESET voltage) (Fig. 4A). Decreasing the diameter Φ of the BEC from 190 to 80 nm, the RESET energy reduces to 0.27 and 2.10 nJ, respectively. We partly attribute this dramatic reduction in power consumption by more than 87% to the partial melting in the PCH device, where $\sim 40\%$ of the PCH film is crystalline TiTe_2 and stays unchanged. The confinement TiTe_2 layers are also thermally resistive, suppressing the through-plane heat loss during programming. The RESET operation of the PCH device can be completed on the nanosecond level under low bias, which reduces the RESET energies to the picojoule level. Improved power consumption was also reported in a $\text{GeTe}/\text{Sb}_2\text{Te}_3$ superlattice (42), although its switching mechanism is still under active debate (43, 44).

We improved both the SET time and SET voltage of the PCH device over the GST device (Fig. 4B and fig. S10). In particular, the PCH device reached ~ 8 ns SET time at ~ 1.5 V, which fulfills the sub-10-ns speed and the low-bias requirements for DRAM-like applications. In comparison, at ~ 1.5 V the GST device needed ~ 80 ns for SET operations. Similar to RESET operations, the enhanced thermal efficiency by

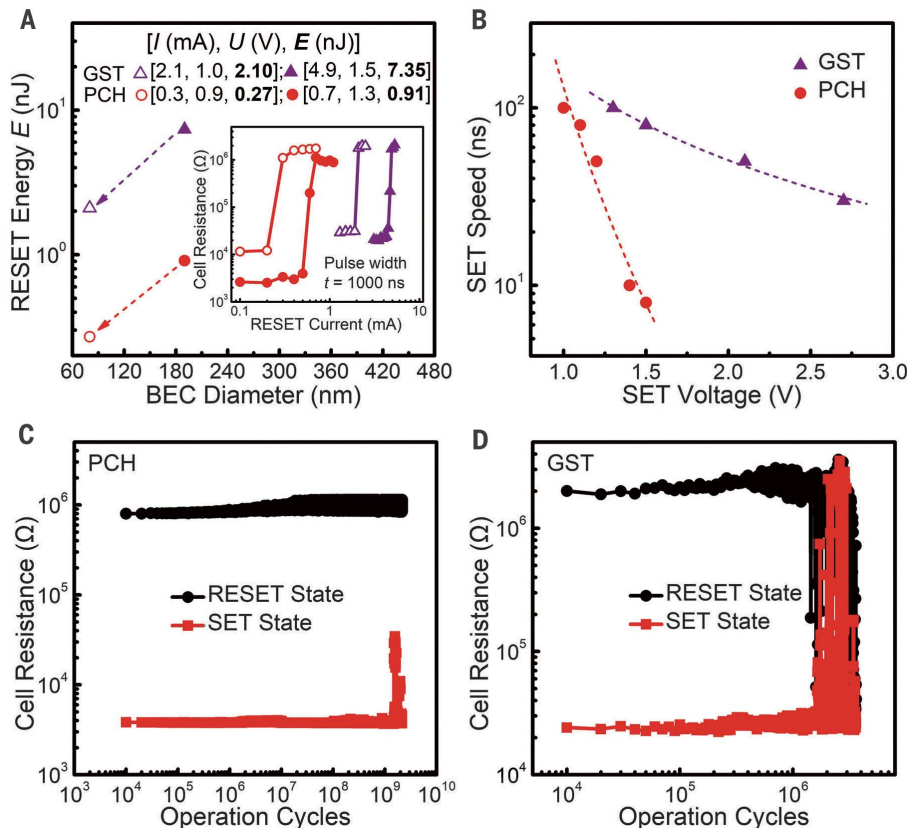


Fig. 4. Improved RESET energy, SET speed, and cycling endurance. (A) RESET energy (E) as a function of BEC diameter for the GST and PCH devices. (Inset) Cell resistance versus RESET current (I) curves with a fixed pulse width of 1000 ns (t) for both devices with different BEC ($\Phi = 80, 190$ nm). Transient RESET voltage (U) across the device is recorded once the RESET state is reached. The input RESET energy (E) is calculated as $E = I \times U \times t$. E depends on t , which can be reduced to subnanosecond level, resulting in picojoule-level RESET energies. (B) SET speed as a function of voltage bias for PCH and GST devices with the same geometry (190 nm in BEC diameter). The PCH device can accomplish SET operations within 8 ns at 1.5 V. (C) Approximately 2×10^9 cycling endurance of the PCH device: SET (at 1.7 V) and RESET (at 2.3 V) with 10-ns voltage pulses. (D) Approximately 1×10^6 cycling endurance of the GST device: SET (at 2.5 V) and RESET (at 5.0 V) with 50-ns voltage pulses.

the confinement layers and the partial switching manner of the memory cell work together to reduce the SET time, bias, and energy. Moreover, the TiTe_2 layers have a similar atomic arrangement as that of crystalline Sb_2Te_3 and may serve as potential heterogeneous nucleation sites to facilitate the crystallization of Sb_2Te_3 at elevated temperatures. The enhanced crystallization tendency is evidenced by the fact that a similar device with Sb_2Te_3 film (~ 150 nm) took ~ 60 ns to complete the SET operations at ~ 1.5 V and needed ~ 4 V to reach ~ 8 ns (9). This difference indicates a reduced energy barrier for crystallization in the presence of the TiTe_2 layers. Our DFT energy analyses also suggest favorable interfacial interaction for crystallization between the crystalline TiTe_2 slab and the amorphous Sb_2Te_3 slab (fig. S5).

Regarding the cycling endurance, extensive cycles of high-bias voltage pulses would trigger long-distance atomic migrations of Sb (Ge) and Te elements in opposite (vertical) directions, giving rise to elemental segregation as well as void formation near the bottom electrode (11). The best-performing PCH device showed a cycling endurance of $\sim 2 \times 10^9$ (Fig. 4C), which is three orders of magnitude longer than the $\sim 1 \times 10^6$ cycles of the GST device with the same geometry (Fig. 4D). Additional examples consistently showing low noise and endurance up to 10^8 to 10^9 cycles are displayed in fig. S11. We attribute the extended cycling life to the smaller bias and energy requested for rapid programming of the PCH device and the effective role of the rigid and dense TiTe_2 walls in reducing the possibility of the long-range elemental diffusion along the pulsing direction. The intrinsic materials innovation offered by our PCH architecture should be easily integrated with other extrinsic device and programming strategies. Examples of the latter include the shrinking down of the switching volume by using a fabrication line of industrial quality that can increase the cycling endurance of GST devices to $\sim 10^{12}$ cycles (45) and programming schemes that trigger self-healing (backward elemental migration) of degraded GST devices upon reversing the polarity of programming pulses (46).

Conclusion

We have demonstrated an innovative design of PCH architecture that offers multiple performance benefits over traditional PCRAM devices

—in particular, the substantially reduced noise and drift in resistance states. The advantages originated from the suppressed compositional and structural variability in phase transition cycles and enabled well-controlled iterative RESET and cumulative SET operations. The PCH approach is amenable to industrial production because the multilayer deposition does not substantially increase the fabrication cost or demand complex procedure, making it relatively easy to be incorporated into state-of-the-art device setups for applications such as high-performance neuro-inspired computing. In addition, our discovery opens the door for nonvolatile DRAM-like memories (9) with stable multilevel storage capacity. Besides potential applications in memory and computing, many two-dimensional transition-metal dichalcogenides are topologically nontrivial (47), with antimony telluride being a prototypical topological insulator (48). Alternate stacking of these nanolayers to form heterostructures (49) may lead to new physics phenomena that are also worth exploring.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S11
References (50–60)

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MATERIALS SCIENCE

Torsional refrigeration by twisted, coiled, and supercoiled fibers

Run Wang^{1*}, Shaoli Fang^{2*}, Yicheng Xiao¹, Enlai Gao^{2,3}, Nan Jiang^{2,4}, Yaowang Li⁵, Linlin Mou¹, Yanan Shen¹, Wubin Zhao¹, Sitong Li¹, Alexandre F. Fonseca⁶, Douglas S. Galvão⁶, Mengmeng Chen¹, Wenqian He¹, Kaiqing Yu¹, Hongbing Lu⁷, Xuemin Wang^{7,8}, Dong Qian⁷, Ali E. Aliev², Na Li^{2,9}, Carter S. Haines², Zhongsheng Liu¹, Jiuke Mu², Zhong Wang², Shougen Yin¹⁰, Márcio D. Lima¹¹, Baigang An¹², Xiang Zhou¹³, Zunfeng Liu^{1†}, Ray H. Baughman^{2†}

Higher-efficiency, lower-cost refrigeration is needed for both large- and small-scale cooling. Refrigerators using entropy changes during cycles of stretching or hydrostatic compression of a solid are possible alternatives to the vapor-compression fridges found in homes. We show that high cooling results from twist changes for twisted, coiled, or supercoiled fibers, including those of natural rubber, nickel titanium, and polyethylene fishing line. Using opposite chiralities of twist and coiling produces supercoiled natural rubber fibers and coiled fishing line fibers that cool when stretched. A demonstrated twist-based device for cooling flowing water provides high cooling energy and device efficiency. Mechanical calculations describe the axial and spring-index dependencies of twist-enhanced cooling and its origin in a phase transformation for polyethylene fibers.

Compared with conventional vapor-compression refrigerators, a solid that changes entropy when deformed could possibly provide higher efficiency; lower cost, weight, and volume; and more convenient miniaturization. An ordinary rubber band is an example, which was reported in 1805 to heat when stretched and to cool when stretch is released (1). Alternative materials for refrigeration are electrocaloric (2–6) and magnetocaloric (6, 7), meaning they provide cooling because of changes in electric or magnetic fields, respectively. Although these solid-state cooling mechanisms have been widely investigated for diverse materials, none yet meets the performance needs for wide-scale applications. In fact, thermoelectrics are the only solid-state coolers that have been commercialized, and these coolers are expensive and have low energy conversion efficiencies.

Efficiencies of ~60% of the Carnot efficiency can be obtained for vapor-compression refrigerators (8, 9), which is a mature technology that typically uses gases whose release can powerfully contribute to global warming. No alternative technology presently achieves this same efficiency. On the other hand, theoretical efficiencies of ~84% of the Carnot efficiency have been predicted for tensile deformation cycles of a NiTi shape memory alloy (9). Realization of close to this efficiency could have enormous consequences if the needed cycle

life of vapor-compression refrigerators could be obtained, given that so much of global energy consumption goes to cooling.

Refrigeration materials that undergo cooling because of any type of mechanical deformation are called mechanocaloric, and those that cool because of changes in uniaxial stress (5, 6, 9–15) or hydrostatic pressure (6, 15–17) are more specifically called elastocaloric and barocaloric, respectively (6). In this study, we demonstrate cooling by a change in yarn or fiber twist, which we call twistocaloric cooling. We call coolers that use twist changes for refrigeration “twist fridges.”

Twist-based cooling using single natural rubber fibers

Vulcanized natural rubber (NR) fibers (18) (figs. S1 to S10) provide a prototypical twistocaloric material. Twisting these fibers causes coil nucleation and propagation, decreasing intercoil separation, and ultimately nucleation of supercoils (Fig. 1A). For isometric strains (i.e., constant strains during twist) of 100, 250, 300, and 450%, fracture occurs before completion of supercoiling, initiation of supercoiling, completion of coiling, and initiation of coiling, respectively. Unless otherwise stated, (i) stresses, strains, and twist densities are relative to the cross-sectional area or length of the nondeformed fiber (called the parent fiber), and (ii) a tensile strain rate of 42 cm/s and a twist-

ing and untwisting rate of 50 turns/s were used for NR fibers having a nondeformed length of 3.0 cm. Here and elsewhere, tensile strain changes are made either while a fiber or yarn is torsionally tethered or for the nontwisted state.

Twistocaloric surface temperature changes occur inhomogeneously for coiled and supercoiled fibers. Hence, maximum and average surface temperature changes ($\Delta T_{\max}$ and ΔT_{avg} , respectively) are reported. Surface temperatures were measured using a thermal camera, whose accuracy was established by comparison with thermocouple measurements in a temperature-controlled oven on fibers with different twist states (18).

The temperature swing on stretch and stretch release (between heating and cooling temperatures) is important for remotely optically readable strain sensors or mechanothermochromic fibers. For 300% isometric strain, this swing in surface-average temperature was 3.3°, 6.5°, and 7.7°C for highly twisted, coiled, and partially supercoiled fibers, respectively, compared with 2.4°C for a nontwisted fiber (Fig. 1B). The maximum surface temperature swing (fig. S11B) for this modest applied strain was 5.4°, 10.5°, and 12.9°C for the highly twisted, coiled, and partially supercoiled fibers, which is up to 5.4 times that for the nontwisted NR fiber. However, when released from strains >300%, nontwisted NR fibers provided greater surface cooling. Hence, these twisted NR fibers are not very useful for refrigerators that solely use large-magnitude stretch and release.

Examples of cooling by untwist are shown in Fig. 1, C to E; figs. S13 to S18; and movie S1. When twist is released from a supercoiled NR fiber having 100% isometric strain, the maximum (–15.5°C) and average (–12.4°C) surface cooling exceed that for 600% stretch release from a nontwisted fiber (–12.2°C) (Fig. 1B). By releasing twist and then stretch (Fig. 2, A and B), even higher maximum (–16.4°C) and average (–14.5°C) cooling was obtained. Hence, a twist-based cooler at 100% strain can be two-sevenths of the maximum length of the above stretch-based cooler and still provide 2.3°C higher surface-average cooling. Figure S34A and movie S2 illustrate the use of a thermochromic dye-coated NR fiber as a mechanothermochromic material whose color responds to changes in inserted twist.

The heating and cooling during stretch and stretch release of a nontwisted fiber are

¹State Key Laboratory of Medicinal Chemical Biology, College of Pharmacy, Key Laboratory of Functional Polymer Materials, Nankai University, Tianjin 300071, China. ²Alan G. MacDiarmid NanoTech Institute, University of Texas at Dallas, Richardson, TX 75080, USA. ³Department of Engineering Mechanics, School of Civil Engineering, Wuhan University, Wuhan, Hubei 430072, China. ⁴Shenzhen Geim Graphene Center, Tsinghua Shenzhen International Graduate School, Shenzhen 518055, China. ⁵School of Life Sciences, Tsinghua University, Beijing 100084, China. ⁶Applied Physics Department, State University of Campinas, Campinas, SP 13081-970, Brazil. ⁷Department of Mechanical Engineering, University of Texas at Dallas, Richardson, TX 75080, USA. ⁸Department of Mechanical Engineering, Georgia Southern University, Statesboro, GA 30458, USA. ⁹Materials Science, MilliporeSigma, Milwaukee, WI 53209, USA. ¹⁰Institute of Materials Physics, Tianjin University of Technology, Tianjin 300384, China. ¹¹Nano-Science and Technology Center, Lintec of America, Richardson, TX 75081, USA. ¹²School of Chemical Engineering, University of Science and Technology Liaoning, Anshan 114051, China. ¹³Department of Science, China Pharmaceutical University, Nanjing, Jiangsu 211198, China.

*These authors contributed equally to this work.

†Corresponding author. Email: ray.baughman@utdallas.edu (R.H.B.); liuzunfeng@nankai.edu.cn (Z.L.)

approximately independent of fiber diameter (fig. S11A). Also, NR fibers having different diameters but the same elongation have approximately the same dependence of ΔT_{max} and ΔT_{avg} on the product of twist density and stretched fiber diameter (fig. S17). The maximum surface cooling on twist release was essentially the same whether twist insertion was done isometrically (for constant tensile strain) or isobarically (for constant tensile load), as long as the percent stretch obtained after isometric twist release was identical (fig. S19). The NR fiber showed stable heating and cooling during isometric twisting and untwisting up to 15 turns/cm for 750 cycles (fig. S21).

Volume-average cooling for twisted NR fibers

The volume-average cooling upon untwisting a NR fiber was obtained from the cooling energy of this fiber in a water-containing plastic tube (fig. S44A). This cooling energy is the sum of the products of gravimetric heat capacity, mass, and temperature decrease for the water, plastic tube, and NR fiber after internal thermal equilibration of the system (fig. S44B). The volume-average cooling upon untwisting a NR fiber was obtained by dividing the derived cooling energy by the heat capacity and mass of the NR fiber (18). To minimize loss of cooling energy to surrounding air (fig. S12) by decreasing the temperature change of the water, a mass of water 4.63 times that of the NR fiber was used. This small loss of cooling energy was corrected by fitting the long-term temperature dependence of water and container to a standard heat-loss equation (19) and then adding these temperature corrections to the observed curves, so that the loss-corrected temperature curves reach long plateaus (18). The temperature changes for these plateaus were used to obtain the cooling energy and thereby the dependence of volume-average temperature change on twist density change.

Figure 1D compares the surface-average and volume-average temperature changes for isometric twist insertion and release from a 100% stretched NR fiber. For the high twist density most important for applications, where the twist removal time (1.5 s) exceeds the calculated radial thermal equilibration time (0.96 s) (18), the volume-average cooling is 11.3°C and the ratio of volume-average to surface-average temperatures reaches 0.91. This is consistent with nearly complete equilibration in the radial direction.

Although the maximum twistocaloric specific cooling energy obtained (19.4 J/g) is lower than reported for releasing 600% stretch from a nontwisted NR fiber (21.6 J/g) (20), the stretch needed for this torsional cooling is much smaller (100%). At 100% strain, stretch-based cooling would only be 4.2% of that delivered by twistocaloric cooling (18).

Coefficients of performance for twisted NR fibers

Using the above volume-average cooling on isometric twist release, the coefficient of performance (COP) for NR twist fridges was obtained. The COP can be described as the ratio of the cooling energy (the product of the volume-average cooling, the gravimetric heat capacity, and the mass of the fiber) to either the input mechanical energy or the net energy consumed during a mechanical cycle (called

COP_{HC} and COP_{FC} , for a half cycle and a full cycle, respectively) (6). COP_{HC} is useful when device simplicity or miniaturization is more important than increasing efficiency by recapturing part of the input mechanical energy.

Using the stretch release-induced cooling of the nontwisted fiber (Fig. 1B), the volume-average cooling during twist release (Fig. 1D), and mechanical measurements (figs. S7 and S10B), the COPs were obtained as a function of volume-average cooling (Fig. 2C and fig.

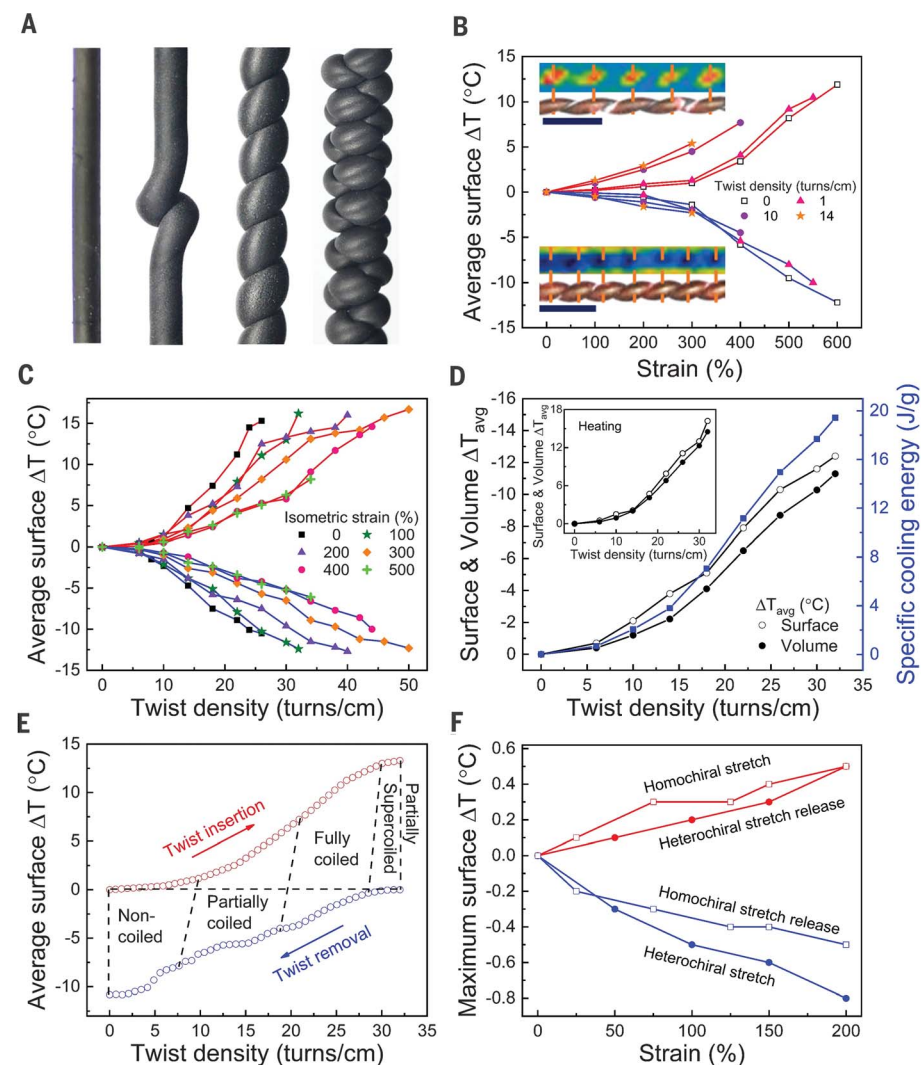


Fig. 1. Twist-based mechanocaloric performance for single rubber fibers. (A) Photographs of twisted, partially coiled, fully coiled, and fully supercoiled 2.5-mm-diameter NR fibers at 100% strain. The background was digitally removed. (B) Surface-average temperature changes versus strain for NR fibers having 0, 1, 10, and 14 turns/cm of twist (nontwisted, highly twisted, fully coiled, and partially supercoiled, respectively, in the nonstretched state). Insets show thermal and optical photographs after 100% stretch (top) and after stretch release (bottom) for a coiled NR fiber (18 turns/cm). Scale bars: 0.5 mm. (C) Surface-average temperature changes of a NR fiber during isometric twist and untwist at different tensile strains. (D) Surface-average cooling, volume-average cooling, and specific cooling energy for isometrically untwisting a NR fiber at 100% strain. Inset shows corresponding results for volume-average and surface-average heating. (E) Surface-average temperature changes for isometrically twisting and untwisting a NR fiber at 200% strain, when measured continuously during an increasing twist and a decreasing twist scan. (F) Twistocaloric temperature changes versus strain for heterochiral and homochiral coiled NR fibers. In the experiments corresponding to (B) to (F), 2.2-mm-diameter NR fibers were used.

S43). The COP_{FC} and COP_{HC} for cooling, either by twist release or by releasing twist and then stretch, are much higher than for cooling by stretch release from the nontwisted fiber when the volume-average cooling is above -0.4°C (Fig. 2C and fig. S43). For the highest volume-average cooling obtained for releasing stretch from a nontwisted fiber (-12.2°C), isometric untwist (-11.3°C), and releasing twist and then stretch (-12.1°C), the COP_{FC} values are 3.8, 8.3, and 8.5, respectively (Fig. 2C), and the COP_{HC} values are 1.9, 5.2, and 5.3, respectively (fig. S43).

The intrinsic efficiency of a material, which is the maximum theoretical efficiency for a refrigerator, is the ratio of COP_{FC} to the COP of a Carnot cycle (COP_{Carnot}). COP_{Carnot} is $T_C/(T_H - T_C)$, where T_C and T_H are the minimum and the maximum temperatures in the cycle, respectively. These material efficiencies for cooling during isometric untwisting and during releasing twist and then stretch are much higher than for cooling during stretch release from a nontwisted fiber, for both full-cycle and half-cycle processes (fig. S43, C and D). For the highest volume-average cooling for

stretch release from a nontwisted fiber, isometric untwisting, and releasing twist and then stretch, the full-cycle intrinsic material efficiencies were 0.32, 0.63, and 0.67, respectively.

Twist-based cooling by plying NR fibers

To demonstrate scalability, cooling was measured while isometrically unplying multiple NR fibers. Unplying seven-ply 2.2-mm-diameter NR fibers (Fig. 2, D and E) produced higher maximum (-19.1°C) and average (-14.4°C) surface cooling than for 600% stretch release from a nontwisted fiber (-12.2°C) (Fig. 1B) or for isometric untwisting of a 100% stretched single fiber (-15.5°C maximum and -12.4°C average) (Fig. 1C and fig. S15). Unplying from isometric strains around 100% produced the highest twistocaloric cooling (figs. S23 and S24).

Guided by the correspondence between twistocaloric cooling for single fibers having the same stretch, and a similar product of twist density and stretched fiber diameter (fig. S17), we found that the twistocaloric cooling associated with isometric fiber plying approximately depends on the product of the twist density of plying and the effective diameter of the bundle ($D_{\text{eff}} = n^{0.5} \times D_s$, where n is the number of fibers and D_s is the stretched fiber diameter) (Fig. 2E).

Spatial and temporal dependencies of twistocaloric temperature changes for NR fibers

Spatially complex surface temperature changes occur for twistocaloric processes for coiled or supercoiled fibers (Fig. 2F and fig. S20). Like for a bent cantilever, the inside of a coiled fiber is compressed and the outside is stretched, causing the coil's exterior to undergo the highest temperature change during coil formation and removal. Painting the exterior of a coiled fiber enabled demonstration that these maximum strained regions have the highest cooling after fiber untwisting. The periodicity of these regions on the surface of the untwisted fiber (5.6 mm) is much longer than the average coil period of the fully coiled fiber (2.5 mm).

The peak surface cooling increases with increasing distance from the site where coiling nucleates (fig. S20), because coiling stretches noncoiled fiber regions and thereby decreases the spring index (the ratio of the average coil diameter to the diameter of the fiber within the coil) of later-introduced coils. The sharp change in maximum surface cooling upon complete removal of coiling (fig. S17A) disappears when measuring average cooling (fig. S17B), indicating that the effects of bending approximately average to zero. Because the number of coils is strain invariant, the separations between temperature peaks during both stretching and releasing are identical to the intercoil separations (Fig. 1B, inset).

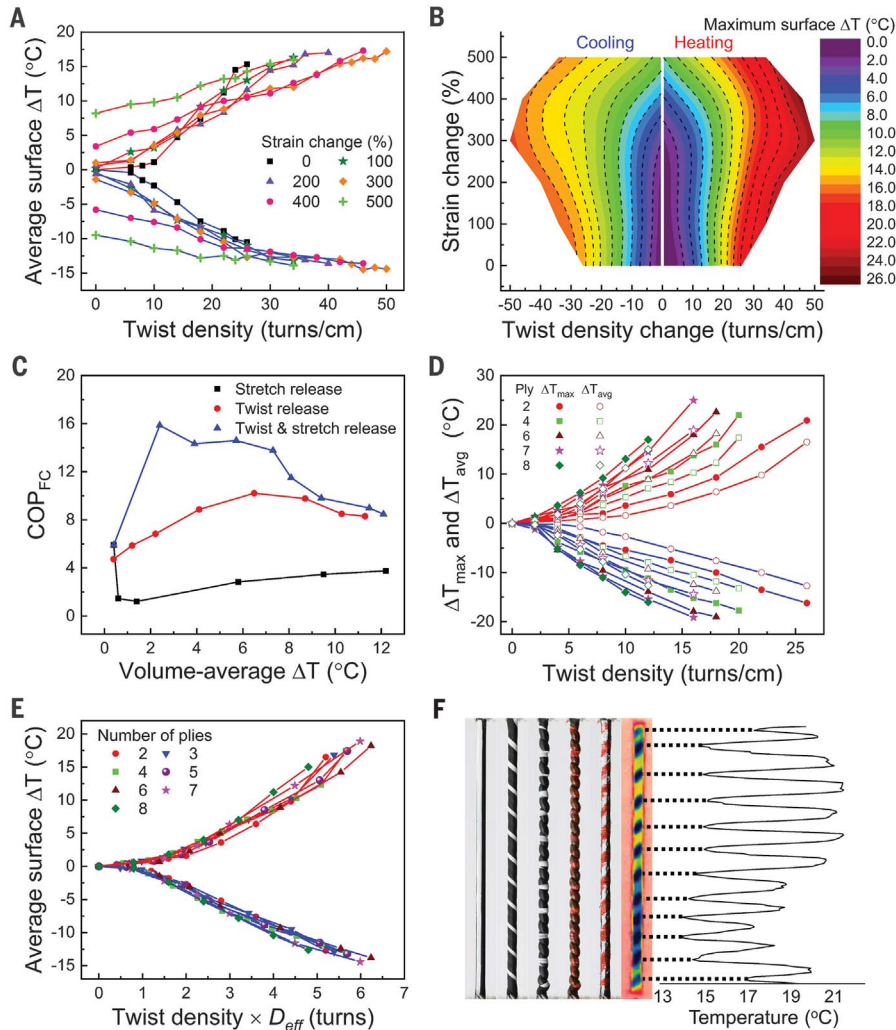


Fig. 2. Mechanocaloric performance of single and plied NR fibers during twist and stretch.

(A) Average and (B) maximum surface temperature changes of a NR fiber during sequential stretch and then isometric twisting and during sequential isometric untwisting and then stretch release. (C) The COP_{FC} versus volume-average cooling for releasing up to 600% strain from a nontwisted fiber (black), isometric untwisting a fiber at 100% strain (red), and isometric untwisting at 100% strain followed by stretch release (blue). (D) The maximum and surface-average temperature changes versus twist density for plying and unplying NR fibers at 100% strain. (E) The surface-average temperature changes of (D) versus the product of twist density and the effective fiber diameter. In the experiments corresponding to (A) to (E), 2.2-mm-diameter NR fibers were used. (F) Photographs of an initially nontwisted, 3-cm-long, 3-mm-diameter NR fiber that was (from left to right) stretched to 100% and painted with a white line along its length; highly twisted; fully coiled; painted red on the coil's exterior; and then fully untwisted. The thermal image and temperature profile (right) show the fiber immediately after removing 12 turns/cm of inserted twist.

Twistocaloric cooling by self-coiled polyethylene and nylon fibers

Twistocaloric cooling was also investigated for polymers used for fishing line and sewing thread. These polymers were made elastically deformable by isobarically twisting until the fiber coils, as is done to make powerful thermally driven artificial muscles (21). These self-coiled fibers are called homochiral because fiber twist and coiling have the same handedness.

Initial experiments used 0.41-mm-diameter, 65-pound test, braided polyethylene (PE) fishing line (fig. S25A). Spring indices from 1.4 to 0.5 were obtained by inserting 6.5 to 7.3 turns/cm of twist under isobaric loads of 37.1 to 74.2 MPa, respectively. When releasing 22.7% strain, maximum and average surface cooling of -5.1°C and -3.2°C , respectively, were obtained for a spring index of 0.8 (Fig. 3A and fig. S25). For comparison, the temperature change upon stretch release of nontwisted polyethylene yarn was below $\pm 0.1^{\circ}\text{C}$, and the highest reported elastocaloric cooling for a nonelastomeric polymer is -2.5°C for a poly(vinylidene fluoride-trifluoroethylene-chlorotrifluoroethylene) terpolymer (15), which is a relaxor ferroelectric (2).

The highest temperature swing between maximum heating and cooling (12.9°C , for a spring index of 0.8) was obtained for 22.7% stretch and stretch release, which is a much higher per-strain change (0.57°C per percent) than for a nontwisted NR fiber (0.04°C per percent for 600% strain). This high sensitivity is useful for mechanothermochromic indicators (movie S3). No degradation in performance occurred during the investigated 2500 stretch-release cycles to 13% strain (fig. S26). The twistocaloric cooling for self-coiled, high-strength polyethylene yarn at 10°C ambient temperature is smaller than for ambient temperatures of 25° and 40°C (fig. S25). In contrast, the stretch-release-induced cooling for a nontwisted NR fiber at 10°C is higher than for either higher or lower ambient temperatures (22).

Twistocaloric cooling was also observed during stretch release for coiled, single-filament nylon 6 fishing line with parent diameters (D) of 0.2, 0.4, and 0.6 mm. To ensure the same spring index (1.0), these fibers were coiled using the same isobaric stress and the same twist number (twist density times D). These homochiral fibers, having progressively larger diameters, provided progressively increasing maximum cooling (-1.3° , -1.9° , and -2.1°C) and average cooling (-0.8° , -1.2° , and -1.8°C) upon stretch release (fig. S28).

The strain dependence of twistocaloric cooling generally increases with decreasing spring index for coiled, high-strength polyethylene yarn (fig. S25), low-strength polyethylene (fig. S27), and nylon 6 fibers (fig. S28). Similarly, for coiled polymer muscles (21), higher heating is needed to cause a given stroke for a smaller spring-index muscle. The origin in both cases is

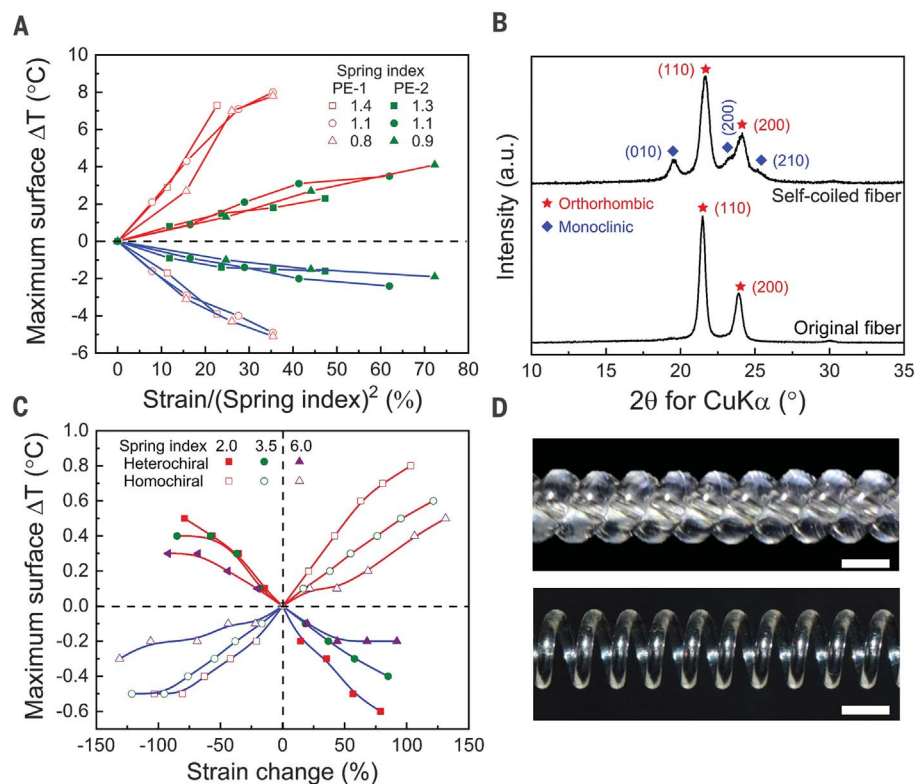


Fig. 3. Twistocaloric performance for coiled polyethylene and nylon 6. (A) The maximum changes in surface temperature versus the ratio of tensile strain to the square of spring index, for high-strength and low-strength polyethylene fibers (PE-1 and PE-2, respectively). (B) XRD for nontwisted and self-coiled PE-1 fibers. (C) Twistocaloric temperature changes versus strain for homochiral and heterochiral coiled nylon 6 fibers having different spring indices. (D) Optical images of a self-coiled, 0.6-mm-diameter nylon 6 fiber (top) and a thermally annealed (180°C for 1 hour), mandrel-coiled, 0.6-mm-diameter nylon 6 fiber (bottom). Scale bars: 1.0 mm (top) and 2.0 mm (bottom).

the stretch-induced twist change per fiber length, whose magnitude is spring-index dependent.

Theoretical results (18) (fig. S39) show that the stretch-induced twist change per fiber length, divided by the percent stretch of a coiled fiber, should approximately depend on the inverse square of spring index. This dependence arises because both the strain needed to pull out one coil and the fiber length per coil linearly increase with coil diameter. In agreement, the observed twistocaloric temperature changes for self-coiled polyethylene and nylon 6 fibers or yarns are approximately proportional to the ratio of percent stretch to the square of the spring index (Fig. 3A and fig. S40). This dependency is predicted only for the case where different-spring-index yarns have approximately the same initial coil bias angle. Owing to the difficulty of controlling this coil bias angle for later-described mandrel-coiled fibers, deviations from these theoretical results are observed.

Origin of twistocaloric cooling of polyethylene fibers

Because the above polyethylene and nylon 6 fibers are crystalline, it is challenging to ex-

plain the origin of the entropy decrease caused by stretching the coiled fiber. One possible explanation for polyethylene is the known deformation-driven orthorhombic-to-monoclinic phase conversion (fig. S41) (23–26). Using molecular dynamics calculations at 300 K (18), we predict that the entropy of the orthorhombic phase is $0.097 \text{ J K}^{-1} \text{ g}^{-1}$ higher than for the monoclinic phase, which agrees with the entropy difference calculated more than 30 years ago (26) from vibrational spectra ($\sim 0.12 \text{ J K}^{-1} \text{ g}^{-1}$). Our predicted free energy difference resulting from this entropy change (29.1 J/g) and the specific heat capacity (27) of polyethylene ($1.56 \text{ J K}^{-1} \text{ g}^{-1}$) provides a predicted -18.7°C cooling if coil stretch caused complete conversion from the orthorhombic to monoclinic phase.

X-ray diffraction (XRD) measurements for polyethylene show that self-coiling results in partial conversion of orthorhombic to monoclinic phase. Thermally annealing the coiled yarn (120°C for 2 hours) largely reverses this transformation (Fig. 3B and fig. S42B) but barely affects twistocaloric cooling (fig. S42A). Stretching the coiled yarn to 20% strain reversibly increases the amount of monoclinic phase from 5.0 to 11.4% (table S2). Using this

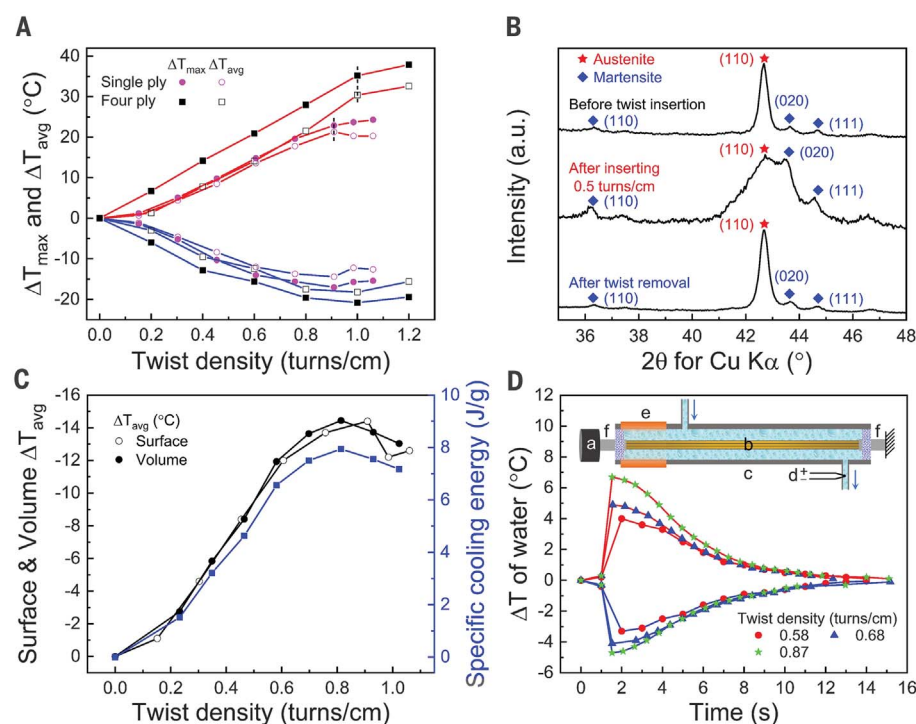


Fig. 4. Twistocaloric performance for twisted and plied NiTi wires. (A) The twist-induced maximum and surface-average temperature changes for a single NiTi wire and four-ply wires at 0% nominal strain. The dashed vertical lines indicate where buckling occurs. (B) XRD for a NiTi wire before twist insertion, after twist insertion, and after twist removal. (C) Surface-average cooling, volume-average cooling, and specific cooling energy for untwisting a 0.7-mm-diameter NiTi wire at 0% strain. (D) The time dependence of outlet water temperature change after isometric twist insertion and twist removal for a three-ply, 0.6-mm-diameter NiTi wire at a water flow rate of 0.04 ml/min using the device in the inset. This device contains (a) a motor, (b) NiTi wires, (c) a polypropylene tube containing flowing water, (d) a thermocouple, (e) a rubber tube, and (f) epoxy resin that seals the tube ends.

percent change of monoclinic phase and the -18.7°C cooling for 100% orthorhombic-to-monoclinic phase conversion, a volume-average cooling of -1.2°C is predicted for 20% strain release, which is identical to the measured surface-average cooling for this strain release. This suggests that monoclinic-to-orthorhombic phase conversion substantially contributes to twistocaloric cooling. The entropy change contribution from the amorphous phase is apparently small, which is consistent with the negligible change in amorphous scattering profile and with the unchanged ratio of the integrated diffraction intensity of the amorphous phase to the total diffraction intensity for the crystalline phases (from 0.49 to 0.50 during 20% stretch).

Twistocaloric cooling by stretching heterochiral fibers

Whereas stretch increases fiber twist for a homochiral coil, stretch causes fiber untwist for a heterochiral coil, which enables reversal of the temperature changes produced by stretch and stretch release. A heterochiral NR coil was made by wrapping a self-coiled 2.2-mm-diameter NR fiber onto a mandrel to

provide a spring index of 2.5 (18). This mandrel was retained during twistocaloric measurements to prevent twist cancellation in the rubber fiber.

This heterochiral NR coil provides an inverse mechanocaloric effect. For 200% strain, the maximum surface cooling during stretch was -0.8°C , and the maximum surface heating upon stretch release was $+0.5^{\circ}\text{C}$ (Fig. 1F and fig. S22A). For comparison, stretching and releasing the same strain from an identical spring index homochiral coil caused maximum surface temperature changes of $+0.5^{\circ}$ and -0.5°C , respectively (Fig. 1F and fig. S22B).

An inverse twistocaloric effect was also found for heterochiral coils of nylon 6 and polyethylene, which can be thermally set to maintain their coiled shape without retention of a mandrel. Stretching heterochiral coils of nylon 6 and polyethylene, with spring indices of 2.0, induces cooling of -0.6° and -0.3°C , respectively, and stretch release produces heating of $+0.5^{\circ}$ and $+0.5^{\circ}\text{C}$, respectively (Fig. 3C and figs. S31 and S32). For comparison, stretching identical spring index homochiral coils of nylon 6 and polyethylene produces a maximum heating of $+0.8^{\circ}$ and $+1.4^{\circ}\text{C}$, respec-

tively, and stretch release produces a maximum cooling of -0.5° and -0.6°C , respectively (Fig. 3C and figs. S29 and S30). When a heterochiral nylon 6 fiber was stretched to 90%, which is beyond the coil's yield stress, the coil irreversibly deformed to form segments having short (1.0 mm) and long (2.8 mm) intercoil periods, which cooled and heated during stretch, respectively (fig. S33).

Twistocaloric cooling by twisting and plying NiTi wires

Large, reversible temperature changes also result from twist insertion and removal from a single NiTi shape memory wire, and from plying and unplying these wires. The investigated 0.7-mm-diameter $\text{Ni}_{52.6}\text{Ti}_{47.4}$ wires had a martensite-to-austenite transition from -29.0° to 15.0°C during heating, and an austenite-to-martensite transition from 13.5° to -44.6°C during cooling (fig. S35B).

Twistocaloric cooling resulted from isometric untwisting a single NiTi wire (-17.0°C maximum and -14.4°C average surface temperature changes) at 0% strain and at a twist rate of 50 turns/s (Fig. 4A and figs. S36 and S37). Unplying a four-ply bundle of NiTi wires produced even higher cooling (-20.8°C maximum and -18.2°C average surface temperature changes), compared with the -17.0°C elastocaloric cooling provided by an identical NiTi wire (table S1). This twistocaloric cooling was stable over 1000 cycles of twisting and untwisting up to 0.6 turns/cm (fig. S38). XRD results demonstrate reversible austenite-to-martensite conversion during twisting and untwisting a NiTi wire (Fig. 4B). Because this conversion is incomplete, there is an opportunity to increase twist-induced cooling by optimizing the NiTi composition and the operating temperature range of the cooler.

The calculated time for internal thermal equilibration of a 0.7-mm-diameter NiTi wire was 2.5 ms (18), which is much faster than the untwist process at 15 turns/s (65 to 455 ms). In agreement, the optically measured surface-average cooling upon untwisting a NiTi wire is close to the volume-average cooling (Fig. 4C) derived from the calorimetric specific cooling energy of this wire (18) (fig. S45). The anomalous behavior at very high twist, where cooling slightly decreases, is likely due to the onset of wire buckling. The maximum specific cooling energy (7.9 J/g) for untwisting the NiTi wire (Fig. 4C) is similar to literature results for stretch-released NiTi wires and sheets (5.0 to 9.4 J/g) and for stretch release of the present wires (9.4 J/g) (18).

A device that enabled one cycle of refrigeration was demonstrated for the cooling of a stream of water (Fig. 4D and figs. S46 and S47). Flowing ambient-temperature water over a three-ply NiTi wire cable, while removing 0.87 turns/cm of plying, cooled the water by

up to -4.7°C ; higher water cooling (-7.7°C) resulted from adding thermal insulation, increasing the water channel diameter, and increasing the water flow rate (18). Integrating the cooling until the water stream returned to ambient temperature provided a specific cooling energy of 6.75 J/g (table S3), indicating that much of the cooling energy of the NiTi wire has effectively chilled the water. The twist-produced temperature changes of a thermochromic paint-coated NiTi wire provided visible indication of torsional rotation (fig. S34B and movie S4).

Summary

Twist release from fibers or fiber plies resulted in high cooling for materials as different as NR and NiTi. The material cooling efficiency was doubled (to 65%) and cooler length was reduced by a factor of two-sevenths by replacing elastocaloric cooling by twistocaloric cooling for NR fibers. A NiTi-based twist-fridge cooled flowing water by up to -7.7°C in one cycle.

Twist induced by stretch release from coiled polyethylene fishing line resulted in surface cooling that was more than 50 times higher than that obtained by releasing stretching from the nontwisted fiber. Depending on the relative chiralities of fiber and coil, polymers were engineered to cool either during stretch or stretch release. The spatial periodicity of surface temperature changes of coiled fibers can be an asset for remotely readable tensile and torsional strain sensors and for color-

changing fibers for fabrics that dynamically respond to body movement.

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SUPPLEMENTARY MATERIALS

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REPORT

SUPERCONDUCTIVITY

Spatial control of heavy-fermion superconductivity in CeIrIn₅

Maja D. Bachmann^{1,2*}, G. M. Ferguson^{3*}, Florian Theuss³, Tobias Meng⁴, Carsten Putzke^{1,5}, Toni Helm¹, K. R. Shirer¹, You-Sheng Li^{1,2}, K. A. Modic¹, Michael Nicklas¹, Markus König¹, D. Low³, Sayak Ghosh³, Andrew P. Mackenzie^{1,2}, Frank Arnold¹, Elena Hassinger^{1,6}, Ross D. McDonald⁷, Laurel E. Winter⁷, Eric D. Bauer⁷, Filip Ronning⁷, B. J. Ramshaw³, Katja C. Nowack^{3,8††}, Philip J. W. Moll^{1,5††}

Although crystals of strongly correlated metals exhibit a diverse set of electronic ground states, few approaches exist for spatially modulating their properties. In this study, we demonstrate disorder-free control, on the micrometer scale, over the superconducting state in samples of the heavy-fermion superconductor CeIrIn₅. We pattern crystals by focused ion beam milling to tailor the boundary conditions for the elastic deformation upon thermal contraction during cooling. The resulting nonuniform strain fields induce complex patterns of superconductivity, owing to the strong dependence of the transition temperature on the strength and direction of strain. These results showcase a generic approach to manipulating electronic order on micrometer length scales in strongly correlated matter without compromising the cleanliness, stoichiometry, or mean free path.

The electronic ground state of heavy fermions sensitively depends on the coupling between a localized state and an itinerant electronic system. As the coupling strength is tuned, metallic, superconducting, or magnetically ordered phases are induced, yielding the rich phase diagrams typical for materials of this class. This tunability could be exploited for device-based applications if the electronic order can be locally controlled within a crystalline sample: Metallic, magnetic, or superconducting regions could be induced within a single crystal by precise spatial control over the tuning parameter. Strain is a particularly powerful way to achieve this goal: It introduces no disorder, and its independent components offer multiple degrees of freedom to couple to electronic order. Most commonly, uniform uniaxial strain (1) or biaxial strain (2) is applied. In this work, we demonstrate micrometer-scale control over the superconducting order in stoichiometric and ultraclean CeIrIn₅ by inducing a nonuniform

tailored strain field in microstructured single-crystal devices. Our experimental approach exploits strain induced by differential thermal contraction between the sample and the substrate, as well as our submicrometer control over the shape of the sample. The tetragonal

heavy-fermion metal CeIrIn₅ exhibits material parameters that are ideal for establishing spatial control of a correlated state [Sommerfeld coefficient $\gamma \sim 720 \text{ mJ mol}^{-1} \text{ K}^{-2}$ (3), effective mass $m^* \sim 30m_e$, where m_e is the mass of an electron (4)]. The superconducting transition temperature, T_c , of this material is highly sensitive to strain, owing to the strong dependence of Ce 4f hybridization on the Ce-Ce interatomic distance. Straining the sample along the a direction increases the bulk superconducting transition temperature ($T_{c0} = 400 \text{ mK}$) by $56 \text{ mK/kbar} \sim 14\% T_{c0}/\text{kbar}$, whereas compression along the c direction decreases it at the rate of $-66 \text{ mK/kbar} \sim -16.5\% T_{c0}/\text{kbar}$ (5, 6). Although uniaxial strain strongly alters T_c , the almost equal but opposite effects of a - and c -direction strain lead to an overall weak change of T_c under hydrostatic pressure ($10 \text{ mK/kbar} \sim 2.5\% T_{c0}/\text{kbar}$) (7). For a crystal subjected to a nonuniform strain field, complex patches of superconductivity are expected to appear within the heavy Fermi liquid.

Figure 1 illustrates nonuniform superconductivity for the simple case of a rectangular slab also referred to as a lamella. The lamella ($150 \mu\text{m}$ by $30 \mu\text{m}$ by $2 \mu\text{m}$) was carved from a macroscopic crystal by using focused ion beam (FIB) machining [for fabrication details, see (8–10)] and was joined to the sapphire substrate with a thin layer of epoxy (approximately

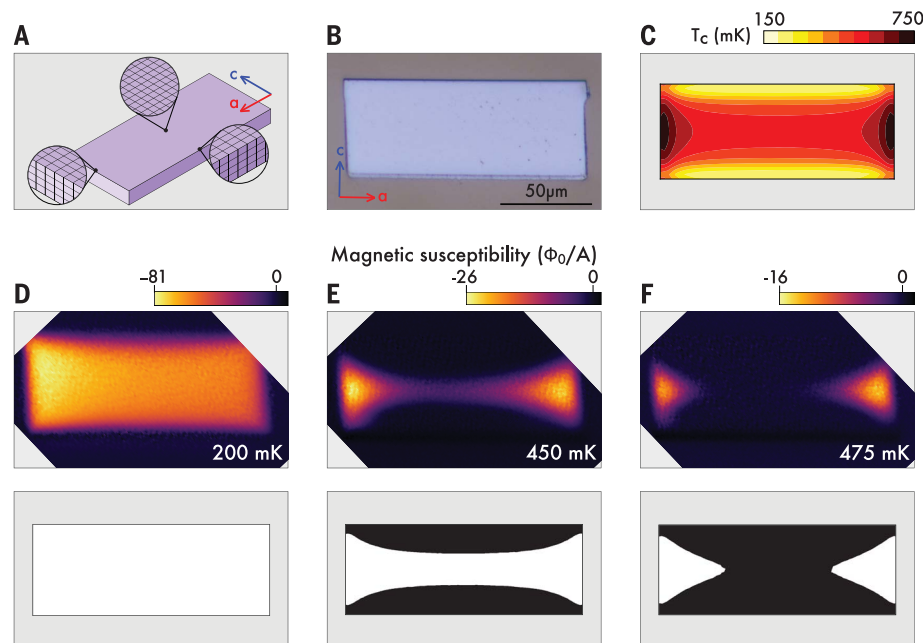


Fig. 1. The superconducting transition in a lamella under biaxial strain. (A) Sketch of the distortion of a thin lamella of CeIrIn₅ coupled to sapphire at low temperatures. (B) Optical image of a 2- μm -thick lamella cut by FIB machining in the (a , c) plane. (C) Finite element simulation of the T_c map across the sample, arising from the strain profile and strain dependence of T_c (10). (D to F) (Top) Local susceptibility images at three representative temperatures. A negative diamagnetic susceptibility indicates superconducting regions of the sample. The susceptibility is measured in units of superconducting magnetic flux quanta (Φ_0) detected in the SQUID per ampere (A) applied to the field coil. (Bottom) Superconducting regions (white) calculated from the strain profile in the device, corresponding to constant temperature contours of the T_c map in (C).

¹Max Planck Institute for Chemical Physics of Solids, D-01187 Dresden, Germany. ²School of Physics and Astronomy, University of St. Andrews, St. Andrews KY16 9SS, UK. ³Laboratory of Atomic and Solid State Physics, Cornell University, Ithaca, NY 14853, USA. ⁴Institute for Theoretical Physics, Technical University Dresden, D-01062 Dresden, Germany. ⁵Institute of Material Science and Engineering, École Polytechnique Fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland. ⁶Physik-Department, Technische Universität München, Garching, D-85748 Germany. ⁷Los Alamos National Laboratory, Los Alamos, NM 87545, USA. ⁸Kavli Institute at Cornell for Nanoscale Science, Cornell University, Ithaca, NY 14853, USA.

*These authors contributed equally to this work.

†These authors contributed equally to this work.

††Corresponding author. Email: philip.moll@epfl.ch (P.J.W.M.); kcn34@cornell.edu (K.C.N.)

a few hundred nanometers thick). This epoxy is substantially softer than the crystalline substrate and the sample; finite element modeling corroborates the intuitive assumption that the differential thermal contraction transmitted through the epoxy is largely independent of the exact details of the glue layer, such as its thickness or elastic moduli [see (10) for details]. The crystallographic c direction is aligned with the short side of the lamella and the a direction with the long side. The use of sapphire cut along the (0001) surface ensures isotropic thermal contraction of the substrate. Whereas sapphire is known for its low thermal contraction, CeIrIn₅ contracts strongly upon cooling, as is typical of many Ce-based compounds (11). As a result, the sample is under tensile strain at low temperature.

To study the superconducting transition in the lamella, we use scanning superconducting quantum interference device (SQUID) micros-

copy (SSM) to image the diamagnetic response of the sample with micrometer-scale resolution. To detect superconductivity, we apply a local magnetic field by sourcing a current through a $\sim 6\text{-}\mu\text{m}$ field coil integrated on the SQUID chip. The $\sim 1.5\text{-}\mu\text{m}$ SQUID pickup loop detects the local magnetic susceptibility measured in magnetic flux per unit current in the field coil [see (10) for details]. Superconducting regions of the sample exhibit a strong diamagnetic response, which enables us to distinguish them from metallic and insulating regions.

Susceptibility images as a function of temperature (top images in Fig. 1, D to F) reveal that superconductivity first emerges at the short edges while most of the lamella remains metallic. As the temperature is lowered, larger fractions of the lamella become superconducting, thus leading to the growth of triangular superconducting patches protruding into the

lamella, which eventually join in the center (Fig. 1E). At even lower temperatures, the order parameter remains suppressed on the long edge. The observation of superconductivity at the edge of the sample before the interior is unexpected. Usually, for a thin superconducting slab cooled in Earth's magnetic field, the demagnetization factor at the sample edges initially favors the appearance of superconductivity in the center of the sample (12).

Before analyzing the spatial pattern in the images, we estimate whether strain caused by differential thermal contraction combined with the strain sensitivity of T_c in CeIrIn₅ can cause the observed variations in T_c of several hundred millikelvin. When cooled to cryogenic temperatures, CeIrIn₅ and sapphire contract by ~ 0.3 and $\sim 0.08\%$, respectively. Given this mismatch, we expect strain on the order of 0.1% to exist within the CeIrIn₅ crystal at low temperature. Using a typical elastic modulus

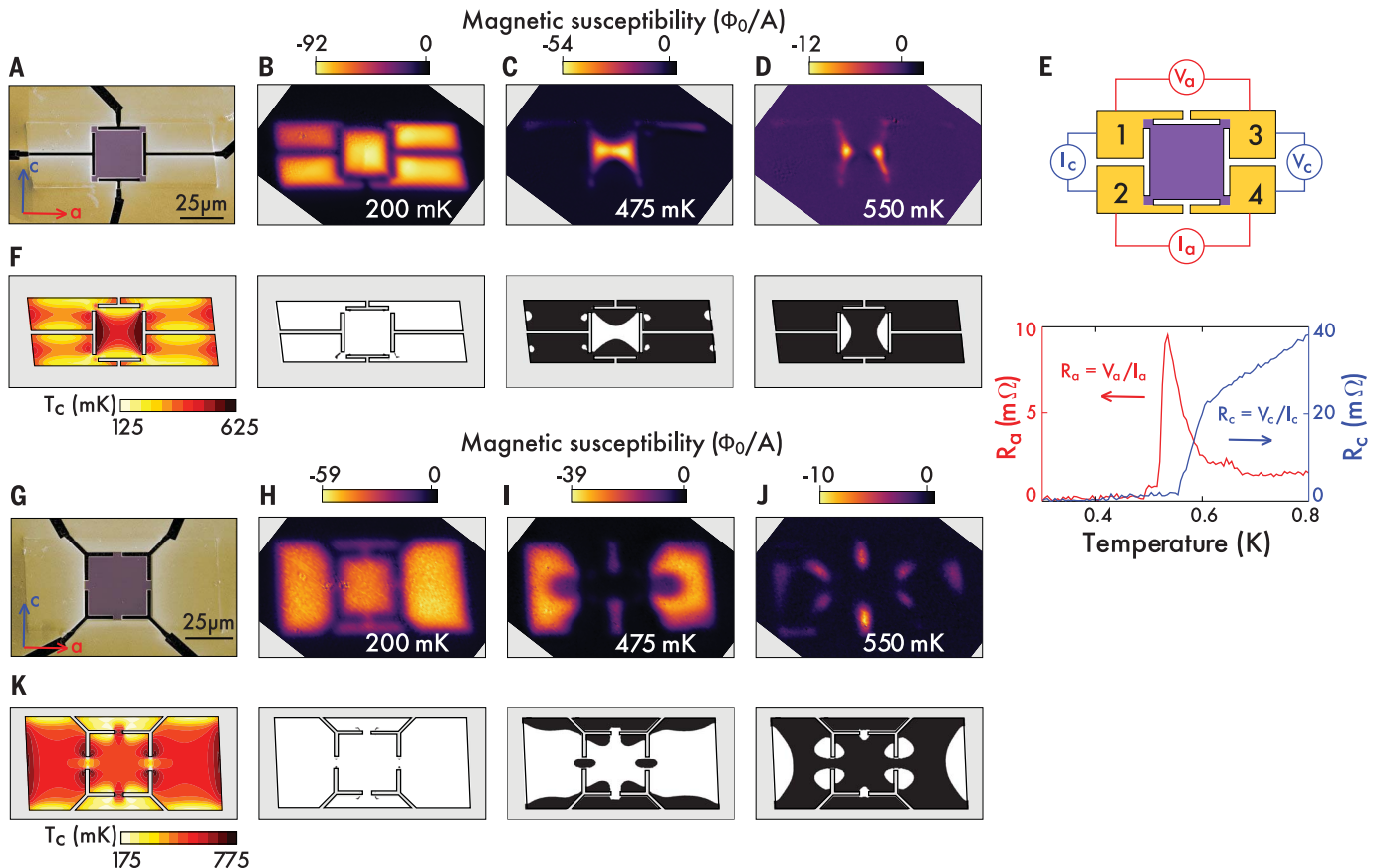


Fig. 2. Spatial control over correlations. (A) Scanning electron microscopy (SEM) image of device 1. The lamella is FIB-cut in the (a , c) plane and contacted by evaporated Au (yellow). The center square of the device is $25\text{ }\mu\text{m}$ by $25\text{ }\mu\text{m}$ by $3\text{ }\mu\text{m}$, held by contacts in the corners. (B to D) (Top) Local susceptibility images at three representative temperatures illustrate the temperature evolution of the spatially modulated superconducting state. (Bottom) Calculated superconducting patterns (10). (E) Montgomery transport measurement upon cooling of device 1. As the first superconducting regions appear on the sides along the c direction (D), the c -direction resistance $R_c = V_c/I_c$ vanishes and the a -direction

resistance $R_a = V_a/I_a$ experiences a resistance spike, caused by a sudden current redistribution. I , current; V , voltage. At lower temperatures, these regions touch (C), leading to zero resistance across all contacts. (F) T_c map for device 1 from finite element calculations (10). (G) SEM image of device 2. The fabrication of device 2 was as similar as possible to that of device 1, but with the constrictions connecting at the middle of each side of the square, not the corners [compare with (A)]. (H to K) The same as in (B) to (D) and (F), but for device 2. A completely different superconducting pattern develops, as expected from the difference in position of the contacts.

of 150 GPa, this is equivalent to a uniaxial pressure of ~ 1.5 kbar, which changes T_c by ~ 100 mK (5). Hence, both the uniaxial pressure studies and the image series presented here are consistent with $\sim 0.1\%$ strain generating ~ 100 mK variation in T_c .

To understand the patterns of superconductivity, we perform finite element method simulations of the device's strain field, which is caused by the difference in the thermal contraction between CeIrIn₅ and sapphire (10). We then compute the local transition temper-

ature, T_c , from the strain field using

$$T_c = T_{c_0} + \frac{\delta T_c}{\delta \epsilon_a}(\epsilon_a + \epsilon_b) + \frac{\delta T_c}{\delta \epsilon_c} \epsilon_c$$

for each point on the grid, resulting in a spatial T_c map (Fig. 1C). Here, ϵ_i with $i = a, b, c$ are the diagonal elements of the strain tensor along the corresponding crystallographic directions. We estimate $\frac{\delta T_c}{\delta \epsilon_a} = -57$ K and $\frac{\delta T_c}{\delta \epsilon_c} = 66$ K from reported bulk measurements of T_c as a function of uniaxial pressure, as well as our measured elastic moduli (5, 6, 10). We

generate binary images from this map, marking superconducting and metallic regions at each temperature that can be directly compared to our susceptibility images (Fig. 1, D to F). We find detailed agreement between the spatial patterns of superconducting regions observed in the SSM images at various temperatures and the pattern predicted by the simulations, which are free of fitting parameters. This agreement indicates that our modeling captures both the physical origin of the complex superconducting patches and the essence of the T_c modulation in the sample. Note that shear strains are not relevant to determine T_c for two reasons: (i) The shear strains are an order of magnitude smaller than the uniaxial strains in the lamella, and (ii) the symmetry of the superconducting order parameter in CeIrIn₅ requires that shear strain couples to T_c at higher order than the uniaxial strains [see (10) for details]. Comparison of the data and simulations shows that including these higher-order couplings is not required to model the experimental results.

Next, we show that the induced strain field and the shape of the superconducting regions can be tailored by using FIB micromachining. To define the strain field in the devices, additional trenches were cut through the lamella down to the substrate, changing the boundary conditions for the elastic equations. In both devices shown in Fig. 2, the trenches define a square in the (a, c) plane. In device 1, the square is anchored by four constrictions, one in each corner (Fig. 2A). In device 2, the constrictions connect to the center of each side of the square (Fig. 2G). Under biaxial tension, each of the contact pads is pulled outward, subjecting the square to nonuniform strain.

Device 1 is designed to measure anisotropic resistances in the plane by passing current through any pair of neighboring contacts while measuring voltage across the remaining pair (13, 14). In this device, the simulated T_c map predicts a pattern of superconductivity first developing on the edges aligned with the c direction as the device is cooled (Fig. 2F). These regions extend toward the center upon lowering the temperature, eventually connecting in the middle of the device. Such patterns are evident in the SSM images (Fig. 2, B to D) and lead to three distinct regimes for transport through device 1: (i) the normal state in which all contacts are separated by metallic regions, (ii) a state in which only the contact pairs along the c direction are connected by superconducting regions, and (iii) a state in which all contacts are connected by a single superconducting region. As a result, when current is sourced between contacts along the c direction (1 and 2), a transition to zero voltage, signaling superconductivity at a relatively high temperature T_c^* , is

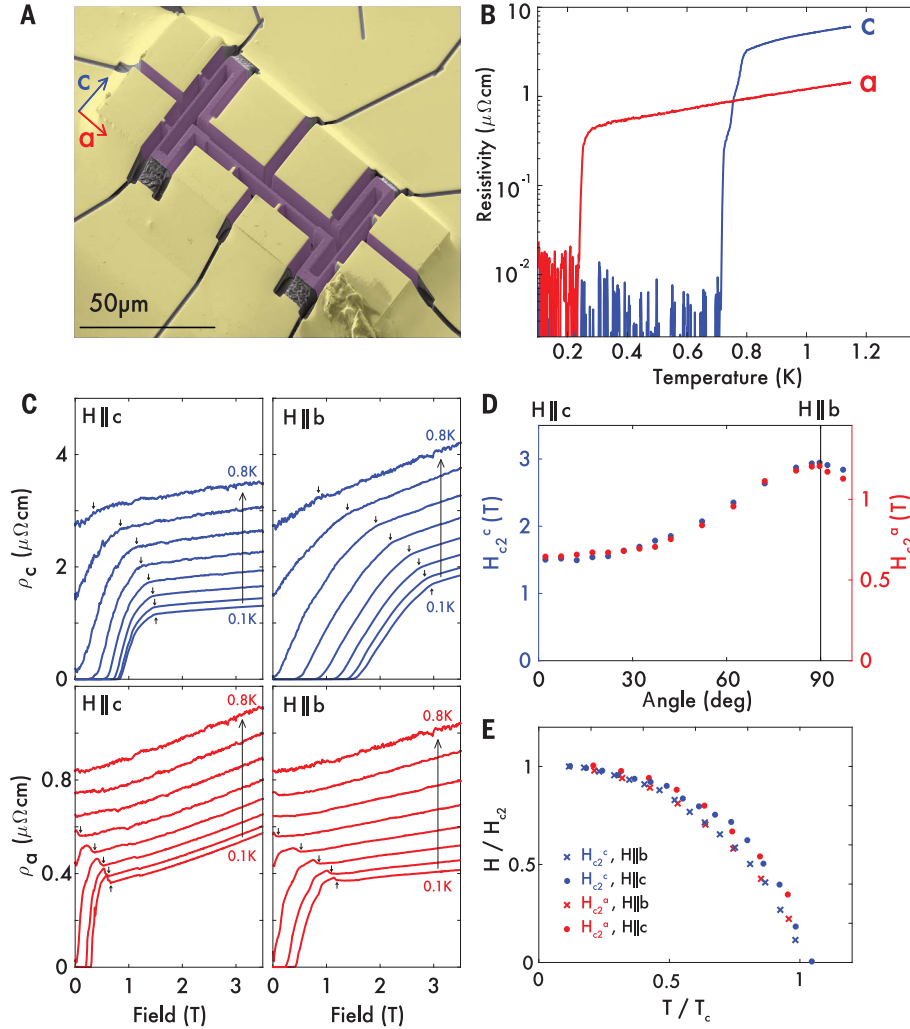


Fig. 3. Smaller structures. (A) SEM image of device 3. The device consists of long bars with dimensions $1.8 \mu\text{m}$ by $8 \mu\text{m}$ by $22 \mu\text{m}$. Two bars are oriented along the c direction and one along the a direction. (B) Resistivity as a function of temperature for device 3. Strain suppresses T_c along the bar aligned with the crystallographic a axis and enhances T_c along bars aligned with the c axis. (C) Temperature-dependent magnetoresistance along the a and c directions in CeIrIn₅ for different field orientations. All measurements were taken in a full Lorentz force configuration except ρ_c for $H || c$, which is naturally longitudinal (ρ , resistivity, H , magnetic field). (D) Angle dependence of the critical fields defined as the points where deviation from the normal magnetoresistance occurs [arrows in (C)], for both current configurations. The data collapse onto a single curve by scaling (compare the two vertical axes). (E) Temperature dependence of the critical fields for all four current and field configurations. The curves for each field configuration collapse when scaled by the respective values of $H_{c2}(0 \text{ K})$ and T_c , using the experimental values for in-plane transport ($T_c \sim 0.45$ K, $H_{c2}^b || b(0 \text{ K}) \sim 1.2T_c$, $H_{c2}^c || c(0 \text{ K}) \sim 0.65T_c$) and along the c direction ($T_c^* \sim 0.8$ K, $H_{c2}^{*c} || b(0 \text{ K}) \sim 2.95T_c$, $H_{c2}^{*c} || c(0 \text{ K}) \sim 1.5T_c$).

observed (Fig. 2E, blue trace). For currents sourced between contacts along the a direction (labeled 2 and 4), we observe a sharp upturn in resistance along a , R_a , as the c -direction resistance, R_c , goes to zero (Fig. 2E). The elongated superconducting regions identified in Fig. 2D cause the current from contacts 2 to 4 to distribute evenly over the width of the device, leading to a larger current to flow at the voltage probes (labeled 1 and 3). Eventually, a second transition to a zero-resistance state occurs when all contacts are connected by a single superconducting region. The electrical response and the direct imaging of this device consistently capture the key concepts of the microscopic control over correlations: Two directed superconducting paths form in a chemically homogeneous metal, arising from the imprinted strain profile.

In contrast to those of device 1, the edges in device 2 parallel to the a axis superconduct well before the central region of the device (Fig. 2, G to K). As with device 1, we find that simulations of the strain profile in the device reproduce the structure of the superconducting transition in detail. The contrast between the two image series indicates that the structure in the images is determined by the interplay between the intrinsic strain sensitivity of the material and the strain field imposed by the FIB-defined features.

In device 2, we observe a pronounced suppression and enhancement of T_c in the a - and c -aligned constrictions, respectively. The strain in the constrictions is enhanced because the contact pads on one side and the square on the other side exert forces on the constrictions that point outward. This observation suggests a strategy to design devices that exhibit a strong modulation of T_c and to generate confined regions of suppressed superconductivity.

Smaller devices exhibit an even more pronounced dependence of the transport T_c on their geometry than larger devices. Device 3 features three series-connected straight beams with dimensions $22\ \mu\text{m}$ by $1.8\ \mu\text{m}$ by $8\ \mu\text{m}$, with two beams aligned with the c direction and one with the a direction (Fig. 3A). These fine structures cannot be resolved in detail with SSM. In transport, the transition temperature for the c -aligned beam $T_c^c \sim 700\ \text{mK}$ is higher than the bulk T_c , whereas the transition temperatures for the a -aligned beams $T_c^a \sim 200\ \text{mK}$ are considerably lower than the bulk T_c . Additional small structures show equally or even more pronounced variations of T_c (10). Therefore, a modulation of the transition temperature by more than a factor of 4 within a single crystal can be realized within our fabrication approach.

To exploit strain tuning of the superconducting order for future device-based experiments, the superconducting state must remain

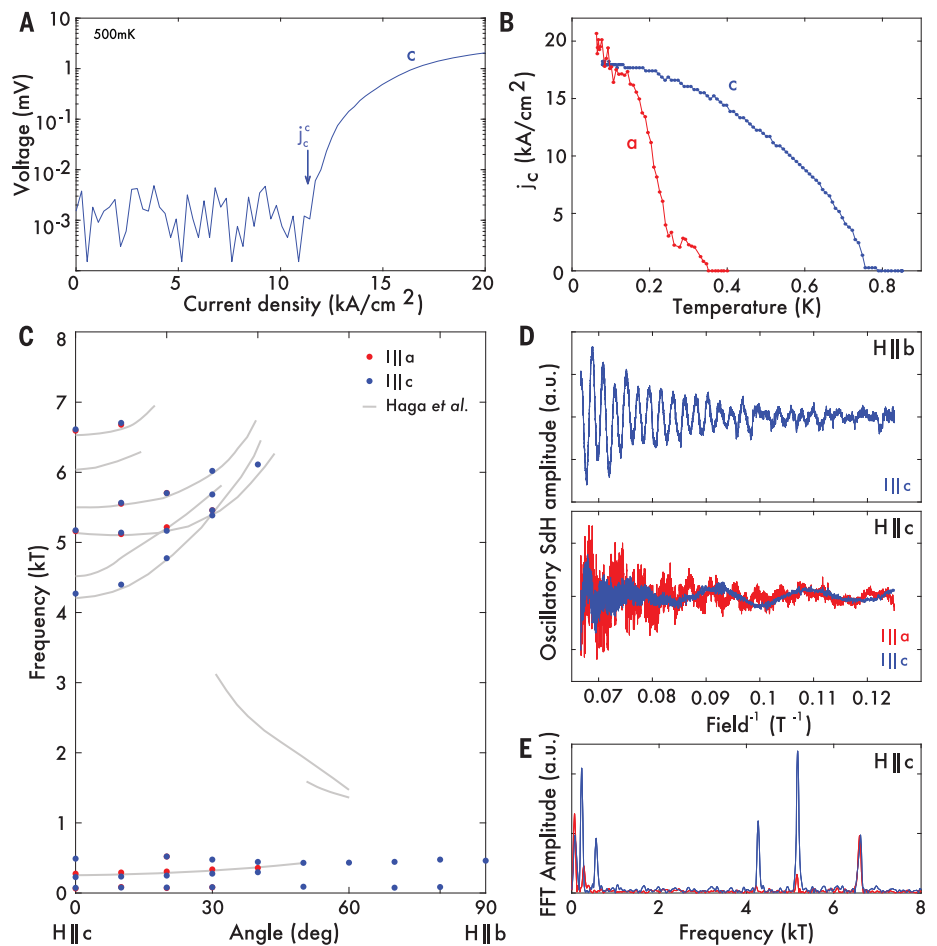


Fig. 4. Robust superconductivity coexists with quantum oscillations. (A) Current-voltage characteristic along the c direction measured on device 3 (see Fig. 3A). The onset of measurable voltage above the experimental noise level (arrow) was used to define j_c^a and j_c^c . (B) Critical current along both directions. A robust, high- j_c state is observed along the c direction, whereas the a direction is in a metallic state. (C) Angle dependence of Shubnikov-de Haas (SdH) oscillations at 80 mK measured in device 3 (points) overlaid on de Haas-van Alphen oscillations measured on bulk single crystals (gray lines) (4). (D) SdH oscillations in the microstructure for $H \parallel b$ (top) and $H \parallel c$ (bottom). a.u., arbitrary units. (E) Fast Fourier transform (FFT) spectrum for the oscillations in (D).

robust after device fabrication. To estimate the critical current of each beam, we apply high currents to device 3 (Fig. 4, A and B). The current is increased until an observable voltage signals the breakdown of the zero-resistance state. To minimize self-heating, 83- μs rectangular current pulses are applied to the sample, with a cooldown time between pulses of 100 ms. Because the critical current decreases monotonically with increasing temperature, the obtained values represent a lower bound of their magnitude in the absence of heating. Figure 4A shows a typical current-voltage characteristic of the c -aligned beam at 500 mK, well above the bulk T_c . A robust zero-resistance state is detected up to a critical current density of $\sim 12.5\ \text{kA}/\text{cm}^2$. Upon cooling, the critical current density increases to $j_c^c(0\ \text{K}) \sim 18\ \text{kA}/\text{cm}^2$ (Fig. 4B). This high critical current is typical for bulk heavy-fermion

superconductors [$\sim 3.8\ \text{kA}/\text{cm}^2$ for UPt_3 (15), $\sim 24\ \text{kA}/\text{cm}^2$ for URu_2Si_2 (15), and ~ 1 to $5\ \text{kA}/\text{cm}^2$ for CeCu_2Si_2 (16)], strongly supporting a scenario of robust, bulk-like superconductivity over the formation of sparse superconducting filaments under strain.

Incidentally, our observations answer an open question about the origin of the commonly observed discrepancy between thermodynamic and resistive measurements of T_c in CeIrIn_5 . Bulk crystals display a transition to a zero-resistance state well above T_c , starting as high as $T_c^* \sim 1.2\ \text{K}$ (17–19) and dependent on the details of the sample. Our results microscopically confirm proposals that strain fields around defects induce this 1 K phase (20–22). In particular, the upper critical fields H_{c2} of the strain-induced superconductivity in the microdevices can be directly scaled onto the bulk values (Fig. 3, C to E). This critical field

scaling was experimentally identified early in the study of macroscopic crystals as a hallmark signature of the 1 K phase (3). On the basis of these observations, we propose that the 1 K phase is a strain effect arising from crystal handling and wire sawing. To directly test this hypothesis, we prepared macroscopic samples by wire saw cutting. Although all samples initially had a $T_c > 1$ K, the resistive signatures of this 1 K phase are completely removed by short surface etching in HCl, after which the resistive transition coincides with thermodynamic probes at $T_c \sim 400$ mK (10). This result demonstrates the absence of defect-strained superconducting patches in single crystals of CeIrIn₅ and establishes the discrepancy between thermodynamic and resistive measurements of T_c in CeIrIn₅ as an effect of surface strain.

Here, we report a strategy to spatially modulate superconductivity within a clean electronic system that is induced by strong, nontrivial strain patterns. Spatial gradients of T_c have been generated by other methods—for example, by modulating the chemical potential across the sample by gradient doping with molecular-beam epitaxy techniques (23). In this method, variations in the local charge carrier density modify the local transition temperature. In the stoichiometric CeIrIn₅ microstructures that we studied, the charge carrier density is uniform, as evidenced by unperturbed quantum oscillations in device 3 (Fig. 4, C to E). These quantum oscillations quantitatively match the angle dependence of previously reported de Haas-van Alphen oscillations (4) measured on macroscopic crystals and indicate that the Fermi surface shape remains unchanged by the weak strain field. In particular, the large, heavy orbits and their fine structure are readily observed, which is usually very difficult in transport. This observation is incompatible with the presence of strong charge carrier density changes across the sample, which would lead to spatial variations in the Fermi surface cross sections and subsequently suppress quantum oscillations by phase smearing.

At the same time, the strain fields in device 3 are strong enough to modulate T_c by almost a factor of 4, from 200 to 780 mK. This finding suggests that the strain field spatially modulates the degree of 4f hybridization across the device and thereby also affects T_c . This mechanism, which is initially surprising, is compatible with experimental observations in the related compound CeRhIn₅ in which hydrostatic pressure suppresses antiferromagnetism and eventually induces superconductivity.

Despite the clear changes in the 4f magnetism and the spin fluctuation spectrum, the quantum oscillation frequencies remain unchanged in the entire pressure range up to the quantum critical point (24). However, the 4f hybridization increases, as evidenced by a quasiparticle effective mass that grows in response to applied pressure. This result strongly suggests that the hybridization with the 4f electrons varies without changes in the overall volume of the Fermi surface. Here we propose that the same microscopic physics underlies the spatial modulation of T_c in CeIrIn₅ microstructures.

In general, strongly correlated materials exhibit a pronounced sensitivity to perturbations, owing to the small energy scales defining their physics. The strain accessible by our fabrication approach is sufficient to substantially alter the electronic properties of these materials without introducing chemical disorder. Unlike chemical approaches to tune correlations, the FIB provides micrometer-scale control over both the direction and magnitude of the induced strain field. We expect that the approach demonstrated here will enable spatial control of a wide range of broken symmetry states in strongly correlated systems. We envision clean interfaces between regions with different electronic order within a sample generated by a spatially modulated strain field—e.g., by generating superconducting regions in structures made from antiferromagnetic CeRhIn₅ (24). Further, this approach is immediately compatible with any material that can be patterned using a FIB. Notably, although we focus on devices aligned along specific crystal axes, devices can be oriented along any direction to generate strain patterns of any desired symmetry. Strain engineering may offer an alternative way to fabricate superconducting circuitry within a metallic layer without any physical junctions, providing a route to fabricating superconductor/normal metal/superconductor Josephson junctions within a single crystal.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Supplementary Text
Figs. S1 to S13
Tables S1 and S2
References (26–37)

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ELECTROCHEMISTRY

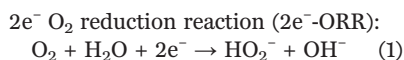
Direct electrosynthesis of pure aqueous H₂O₂ solutions up to 20% by weight using a solid electrolyte

Chuan Xia*, Yang Xia*, Peng Zhu, Lei Fan, Haotian Wang†

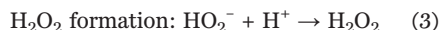
Hydrogen peroxide (H₂O₂) synthesis generally requires substantial postreaction purification. Here, we report a direct electrosynthesis strategy that delivers separate hydrogen (H₂) and oxygen (O₂) streams to an anode and cathode separated by a porous solid electrolyte, wherein the electrochemically generated H⁺ and HO₂[−] recombine to form pure aqueous H₂O₂ solutions. By optimizing a functionalized carbon black catalyst for two-electron oxygen reduction, we achieved >90% selectivity for pure H₂O₂ at current densities up to 200 milliamperes per square centimeter, which represents an H₂O₂ productivity of 3.4 millimoles per square centimeter per hour (3660 moles per kilogram of catalyst per hour). A wide range of concentrations of pure H₂O₂ solutions up to 20 weight % could be obtained by tuning the water flow rate through the solid electrolyte, and the catalyst retained activity and selectivity for 100 hours.

Hydrogen peroxide (H₂O₂) is a nexus chemical for a variety of industries, currently produced through the indirect, energy-demanding, and waste-intensive anthraquinone process (1, 2). This traditional method usually generates H₂O₂ mixtures with concentrations of 1 to 2 weight % (wt %), necessitating further costly purifications and distillations to reach concentrations appropriate for commercial use (3). The overall process requires centralized infrastructure and thus relies heavily on transportation and storage of bulk H₂O₂ solutions, which are unstable and hazardous (4). Direct synthesis of H₂O₂ from a hydrogen (H₂) and oxygen (O₂) mixture (Fig. 1A) provides an alternative route for small-scale on-site generation (5–7). Catalyst development for this reaction has progressed over the past decade (8–12), exemplified by a palladium-tin catalyst with high selectivity (>95%) and productivity [61 moles per kilogram of catalyst (kg_{cat}) per hour] for H₂O₂ synthesis (9). However, a drawback of this route is the inherent flammability hazard associated with mixing high-pressure H₂ and O₂ (13). In practice, the H₂ feedstock must be heavily diluted using CO₂ or N₂ carrier gas, substantially lowering the yields of H₂O₂. In addition, the use of methanol solvent leads to extra purification costs in the preparation of pure aqueous H₂O₂ solutions.

By contrast, electrosynthesis of H₂O₂ can decouple the H₂/O₂ redox exchange into two half-cell reactions (Eqs. 1 and 2), followed by the ionic recombination process (Eq. 3):



H₂ oxidation reaction (HOR):



In the electrochemical process, O₂ and H₂ can be kept safely separated and introduced in pure form to accelerate the reaction. The synthesis can proceed under ambient conditions for on-site H₂O₂ generation and could potentially even output electricity. Although there have been selective catalysts such as noble metals or carbon materials developed for the 2e[−] ORR pathway (14–18), the H₂O₂ product has typically been generated in a mixture, with solutes in traditional liquid electrolytes ranging from acidic to alkaline pH. Extra separation processes to recover pure H₂O₂ solutions were therefore required. Other designs including the use of deionized (DI) water or a polymer electrolyte membrane as the ion-conducting electrolyte have been explored on a preliminary basis for obtaining pure H₂O₂ solutions, but they generally suffered from low reaction rates, product concentrations, or Faradaic efficiencies (FEs) (supplementary text, note 1) (19–21).

Here, we report a porous solid electrolyte design to realize direct electrosynthesis of pure H₂O₂ solutions. As illustrated in Fig. 1B and figs. S1 and S2, independent H₂ and O₂ streams were respectively delivered to HOR and 2e[−]-ORR catalysts coating gas diffusion layer (GDL) electrodes. The anode and cathode “sandwiched” the cation exchange membrane (CEM) and anion exchange membrane (AEM) layers (see materials and methods for details) to avoid flooding by direct contact with liquid water. In the center, a thin and porous solid electrolyte layer facilitated ionic recombination of H⁺ and HO₂[−] ions crossing from the anode and cathode with small ohmic losses; a flowing DI water stream confined to this middle layer could then dissolve the pure H₂O₂

product with no introduction of ionic impurities. By tuning the HO₂[−] generation rate or the DI water flow rate, a wide range of H₂O₂ concentrations (from hundreds of parts per million to tens of percent) could be directly obtained with no need for further energy-consuming downstream purification.

To deliver efficient conversion, electrocatalysts with high activity and selectivity for 2e[−]-ORR and HOR are a prerequisite. We chose the state-of-the-art platinum on carbon (Pt/C) catalyst for HOR at the anode, which affords high H₂-to-H⁺ conversion rates at small overpotentials (22–24). For the cathode, however, electrocatalysts with high activity and selectivity for 2e[−]-ORR toward H₂O₂ have been much less thoroughly explored than the extensively studied fuel-cell catalysts for 4e[−]-ORR to H₂O. Recent studies on noble metal catalysts such as Au-Pd or Pd-Hg (14, 25), as well as carbon materials such as graphene, carbon nanotubes, or porous carbon (15, 16, 26–29), have demonstrated high selectivity toward the 2e[−] pathway. Nevertheless, practical current densities (hundreds of milliamperes per square centimeter) with high FEs, particularly at neutral pH for the purpose of pure H₂O₂ generation, have not yet been achieved. We chose commercial carbon black as the starting material because of its low cost, its high surface area (fig. S3) for high mass activity, and, especially, its nanoparticulate morphology (fig. S3) to facilitate O₂ diffusion from the GDL (layer-by-layer stacking of graphene nanosheets, by contrast, can hinder gas transport). Surface functional groups such as ethers (C–O–C) and carboxylic acids (HO–C=O) have previously been posited to activate the adjacent carbon atomic sites for selective 2e[−]-ORR (15, 16). Hence, we treated the carbon black nanoparticles with nitric acid to introduce such oxidized functionality (see materials and methods and supplementary text, note 2). No morphological changes were observed for these carbon black nanoparticles after acid treatment (fig. S3); however, high-resolution x-ray photoelectron spectroscopy (XPS) (fig. S4) confirmed that acid treatment enriched the particles with oxygen-containing functional groups, including C–O–C/C–OH and HO–C=O, as deconvolved from carbon and oxygen 1s signals.

We found that surface oxidation strongly correlated with H₂O₂ selectivity and activity (fig. S5). The selectivity rose from <80% for the unoxidized particles to ~95% for even relatively low surface oxygen coverage (2.11%). Although the H₂O₂ selectivity was similar upon further increasing the surface oxygen coverage from 2.11 to 11.62% as shown in fig. S5B, we found that the 2e[−]-ORR catalytic activity gradually improved (fig. S5C), which we ascribe to the increased concentration of active sites. After optimization, we selected carbon black with ~10% surface oxygen coverage (CB-10%) as the

Department of Chemical and Biomolecular Engineering, Rice University, Houston, TX 77005, USA.

*These authors contributed equally to this work.

†Corresponding author. Email: htwang@rice.edu

cathode catalyst for further development of the full cell. We first used a standard three-electrode rotation ring-disc electrode (RRDE) system in neutral pH (0.1 M Na₂SO₄) to evaluate the intrinsic activity of CB-10% for benchmark comparisons (see materials and methods). The catalyst presented an impressive H₂O₂ generation performance, with a maximal H₂O₂ selectivity of ~98% and an onset potential of 0.438 V versus reversible hydrogen electrode (RHE), to deliver a 0.1 mA cm⁻² H₂O₂ generation current (fig. S6, A and B). A flow-cell system with GDL electrodes and traditional liquid electrolytes was further used to test the catalyst's performance without O₂ gas diffusion limits in both neutral and alkaline electrolytes (fig. S6C). With a wide potential window to deliver high H₂O₂ selectivity (>90%) in both neutral and alkaline solutions, the catalyst reached maximal FE of 98 and 99%, respectively (fig. S6D), in good agreement with RRDE tests. Furthermore, H₂O₂ partial currents of ~300 mA cm⁻² were achieved, whereas high FEs were still maintained in neutral solutions, better than the highest O₂-to-H₂O₂ conversion rates yet observed (30).

The porous solid electrolyte layer comprises either an anion or cation solid conductor, which can consist of ion-conducting polymers with different functional groups (31), inorganic compounds (32), or other types of solid electrolyte materials such as ceramics, polymer-ceramic hybrids, or solidified gels (33). Among these different solid conductors, polymer ion conductors have been widely used for electrochemistry applications because of their fast ion conduction at room temperature, high reliability, and ease of processing (34). Because proton conduction is generally faster than anion conduction (35), here, we chose to use styrene-divinylbenzene copolymer microspheres (fig. S7), functionalized with sulfonic acid groups for cation (H⁺) conduction (36), as a representative solid electrolyte layer for demonstration. Those copolymer microspheres, once packed together in the middle layer, allow for H⁺ conduction along their interconnected surfaces; in addition, the micrometer pores

formed between these stacked spheres allow for DI water flow and product release (fig. S7). We first studied the impact of the solid electrolyte on H₂O₂ selectivity by the CB-10% catalyst in a standard three-electrode setup (fig. S8A), with potentials calibrated to the RHE scale. The results (fig. S8B) indicate that there were no obvious negative or positive impacts on H₂O₂ selectivity of the CB-10% catalyst when switching from traditional liquid electrolyte to our solid electrolyte. Next, we systematically investigated the H₂O₂ production performance of CB-10% using a two-electrode cell with porous solid electrolyte, as shown

schematically in Fig. 1B. Figure 2A shows the current-voltage (*I-V*) curve of a CB-10%/SE//Pt-C cell with O₂ and H₂ gas streams delivered to the cathode and anode, respectively. The DI water flow rate was fixed at 27 ml hour⁻¹ for this 4-cm² electrode cell to prevent substantial product accumulation, particularly under large currents. H₂O₂ was readily detected starting from a cell voltage of -0.54 V, suggesting an early onset considering the equilibrium voltage of -0.76 V (37). The H₂O₂ selectivity remained >90% across the entire cell voltage range, reaching a maximum of 95% (Fig. 2B). An H₂O₂-generation current of ~30 mA cm⁻²

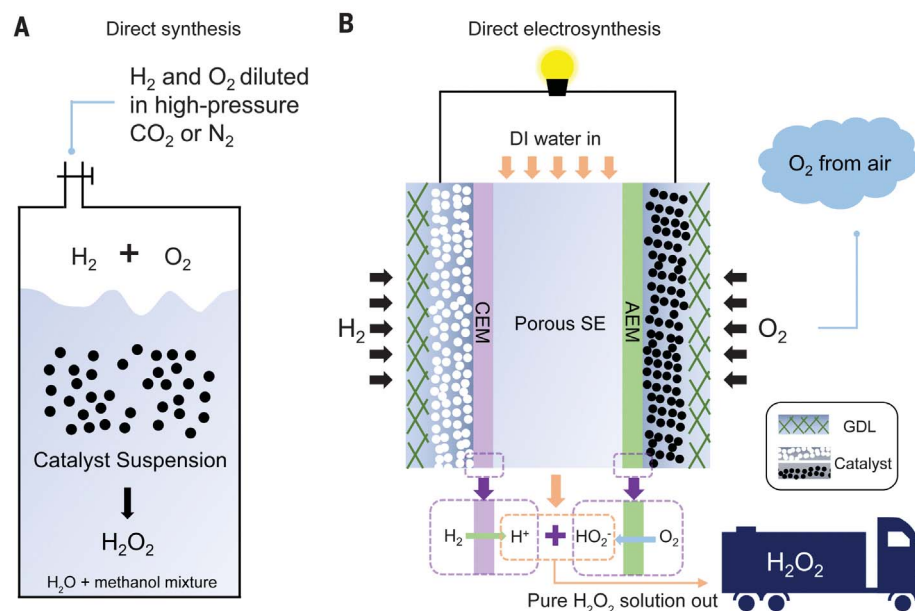


Fig. 1. Schematic illustration of the two different H₂O₂ synthesis methods using H₂ and O₂. (A) Synthesis of H₂O₂ using diluted H₂ and O₂ under high pressure. Methanol used to improve the solubility of the reacting gases in the medium (44) must then be removed downstream. Other studies that avoid alcohols have been performed in acidic solutions of either HCl or H₂SO₄, with NaBr or NaCl as promoters (44). (B) Electrosynthesis of H₂O₂ using pure H₂ and O₂ streams separately introduced to the anode and cathode, respectively. SE represents a solid electrolyte, which consisted in this study of either functionalized styrene-divinylbenzene copolymer microspheres or inorganic Cs₂H_{3-x}PW₁₂O₄₀ (see materials and methods). Electrochemically generated cations (H⁺) and anions (HO₂⁻), driven by the electric field, cross in the porous SE layer and recombine to form H₂O₂. DI water flowing through the porous SE layer then dissolves the H₂O₂ with no impurities.

Table 1. Performance metrics of different H₂O₂ generation methods

	Purity	Productivity (mol kg _{cat} ⁻¹ hour ⁻¹)	Productivity (mmol cm ⁻² hour ⁻¹)	Selectivity (%)	Stability	Max. concentration (ppm)
Our method	Pure	3660	3.4	90 ~ 95	>100 hours	200,000
Direct synthesis	Mixture (8, 9, 45–47)	60.8 ~ 180	N/A	80.7 ~ 96	Up to 4 cycles or 4 hours	5300
Electrochemical synthesis	Mixture (48–53)	N/A	0.05 ~ 1.2	47 ~ 93.5	2 ~ 6 hours	3400 ~ 60,000
	Pure (19–21)	N/A	0.16 ~ 0.289	26.5 ~ 30	6 ~ 72 hours	1400 ~ 80,000

(0.53 mmol cm⁻² hour⁻¹) could be obtained under 0 V (no external energy input). Moreover, a potential of only 0.61 V was required to deliver a current density of 200 mA cm⁻² with an H₂O₂ FE of ~90%. This current represents an H₂O₂-generation rate of 3.4 mmol cm⁻² hour⁻¹, or 3660 mol kg_{cat}⁻¹ hour⁻¹ considering both cathode and anode catalyst (see materials and

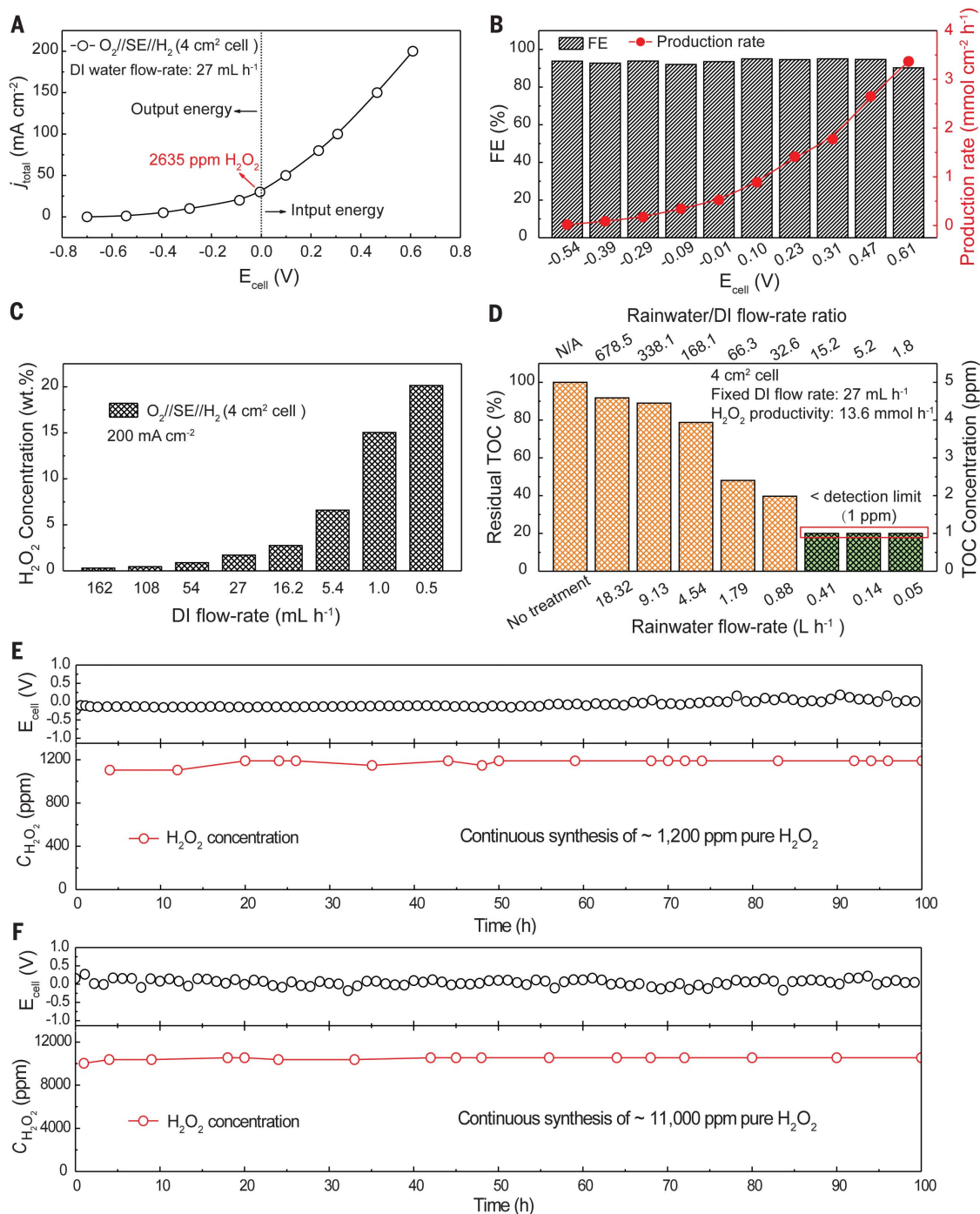
methods; a comparison with literature benchmarks is given in Table 1 and fig. S9). No H₂ byproduct (potentially from H₂ evolution at large overpotentials) was detected from the cathode side under such a high current density (fig. S10A), indicating exclusive selectivity for ORR. Other types of solid electrolyte with different material properties, including poly-

meric conductors for HO₂⁻ conduction and inorganic Cs₂H_{3-x}PW₁₂O₄₀ for cation conduction, were also demonstrated to be effective for pure H₂O₂ solution generation (fig. S11), which suggests the wide tunability and versatility of our solid electrolyte design.

Under a fixed DI water flow rate of 27 ml hour⁻¹, the H₂O₂ concentration from our 4-cm²

Fig. 2. Direct electrosynthesis of pure H₂O₂ using H₂ and O₂ with porous solid electrolyte.

(A) *I*-*V* curve of CB-10%//SE//Pt-C cell with an H⁺-conducting porous solid electrolyte. We define the cell voltage as negative when the cell can output energy during the production of H₂O₂. The positive cell voltage therefore indicates that energy input is required for the reactor. The cell voltages were *iR* (current × resistance) compensated (see materials and methods). **(B)** Corresponding FEs and production rates of H₂O₂ under different cell voltages. **(C)** Dependence of H₂O₂ concentration on the DI water flow rate at an overall current density of 200 mA cm⁻². Up to 20 wt % pure H₂O₂ solutions could be continuously generated for immediate use. The data points in (A) to (C) each represent the mean of two independent measurements. **(D)** Removal of TOC in Houston rainwater using the H₂O₂ solution generated at a fixed current density of 200 mA cm⁻² and a fixed DI water flow rate of 27 ml hour⁻¹ in our 4-cm² electrode device. A high rainwater treatment rate of 0.88 liters hour⁻¹ (0.22 liters cm² electrode hour⁻¹ or 2200 liters m² electrode hour⁻¹) was achieved to meet the drinking water standards (TOC < 2 ppm according to the Texas Commission on Environmental Quality). **(E and F)** Stability tests for continuous generation of pure H₂O₂ solutions with concentrations >1000 and 10,000 ppm, respectively. No degradation of cell voltage or H₂O₂ concentration was observed over the 100-hour continuous operation. The cell currents and DI flow rates were (E) 60 mA and 27 ml hour⁻¹ and (F) 120 mA and 5.4 ml hour⁻¹, respectively.



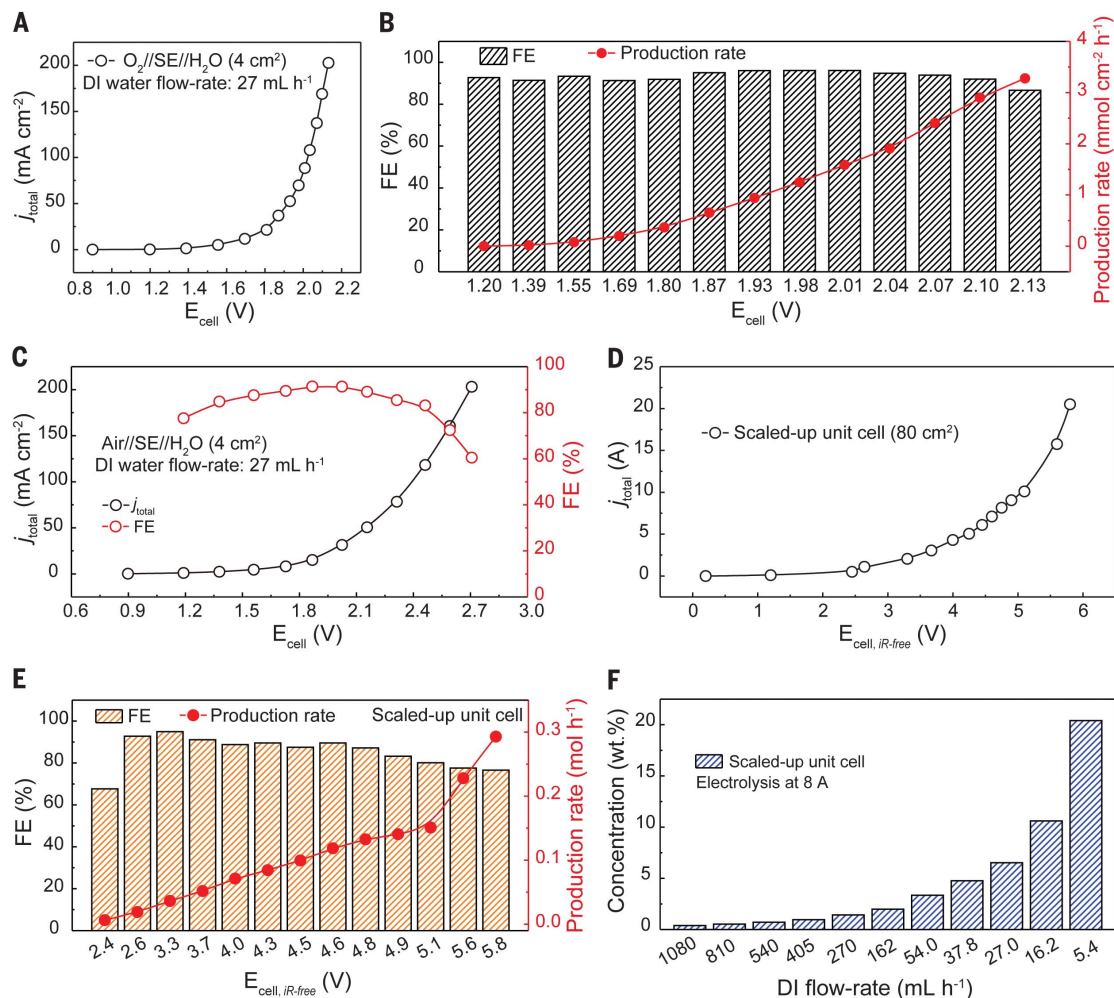
electrode cell reached ~1.7 wt % with an overall cell current of 800 mA. By speeding up or slowing down the DI water flow rate while maintaining the H_2O_2 generation current, we could tune product concentration over a wide range for different application scenarios (Fig. 2C). Up to 20 wt % [200,000 ppm] aqueous H_2O_2 solutions could be directly and continuously obtained by means of electrochemical synthesis. We noticed that the measured H_2O_2 selectivity decreased with increased H_2O_2 concentration (fig. S12A). We ascribe the observed decrease in apparent FE (98% at 0.3 wt % versus 70% at 6.6 wt %) to the following two possible processes. First, the higher concentration of H_2O_2 product in the solid electrolyte layer could shift the equilibrium of the 2e^- -ORR while enhancing the selectivity of the competing 4e^- pathway to H_2O product, thereby lowering intrinsic H_2O_2 selectivity. Second, while H_2O_2 formation proceeds, a fraction of the generated H_2O_2 might not be detected, particularly at high product concentration, because of a potentially increased bimolecular decomposition rate and/or increased crossover to the anode, as frequently

observed in methanol or formic acid fuel cells (38–40); this would result in an apparent decrease in H_2O_2 selectivity. Possible impurities in the product solution, such as sodium (common in water), iron (from the device), sulfur (from the SE), and platinum (from the anode), were quantified to be at or below ppm levels determined by inductively coupled plasma atomic emission spectroscopy (ICP-OES) (table S1 and supplementary text, note 3). Therefore, the electrochemically synthesized H_2O_2 solutions could be put to immediate use out of the cell without any further purification, lowering cost substantially compared with other methods and simplifying the setup for the deployment of on-site generation technology. Long-term stability is another important metric for evaluating catalysis. Our device produced ~1200 and ~11,000 ppm H_2O_2 solutions continuously in 100-hour test runs with no degradation in activity or selectivity (Fig. 2, E and F). XPS characterization of the CB-10% catalyst after the reaction revealed that the surface oxygen functionality was robust and did not appear to have been electrochemically reduced during the operation of the ORR (fig. S10B).

As a representative demonstration of on-site application, we used the as-synthesized H_2O_2 from our device for rainwater purification (Fig. 2D and fig. S13). Compared with traditionally used chlorine compounds, which may produce carcinogens in the processed drinking water (41), H_2O_2 is safe for both human and environmental health when disinfecting and decomposing organic contaminants, typically assessed as removal of total organic carbon (TOC) (42). The use of electrochemically generated H_2O_2 is not only economical (see supplementary text, note 4), but also avoids the transportation and storage of hazardous bulk H_2O_2 . We directly mixed the generated H_2O_2 stream (200 mA cm^{-2} , 27 mL hour^{-1} DI water flow) from our 4-cm^2 electrode device with the rainwater stream (feeding rate ranging from 18.32 to 0.05 L hour^{-1}) to optimize the purification efficiency. The TOC of the pristine rainwater collected at the Rice University campus in Houston was detected to be ~5 ppm (see materials and methods), which is above the Texas treated-water standard of ~2 ppm (43). Decreasing the rainwater feeding rate gradually lowered the TOC remaining (Fig.

Fig. 3. Electrosynthesis of pure H_2O_2 solutions by 2e^- -ORR and water oxidation. (A) I - V curve for an O_2 //SE// H_2O cell in which H_2O is oxidized at the anode side to form protons and O_2 . A 0.5 M aqueous H_2SO_4 solution was used to improve ionic conductivity on the anode side and was not consumed during electrosynthesis.

(B) Corresponding FEs of the O_2 //SE// H_2O cell. (C) I - V curve and FEs for an air//SE// H_2O cell generating pure H_2O_2 solutions. Pure H_2O_2 solutions were generated at a high production rate of $2.3\text{ mmol cm}^{-2}\text{ hour}^{-1}$ ($2490\text{ mol kg}_{\text{cat}}^{-1}\text{ hour}^{-1}$) using only air and water as cathode and anode feedstock, respectively. (D) I - V curve of the scaled-up unit cell module (80 cm^2 electrode, no iR compensation), and (E) the corresponding H_2O_2 FEs. (F) Dependence of H_2O_2 concentration (up to ~20 wt %) on the DI water flow rate at a constant overall current of 8 A. The data points in (A) to (E) each represent the mean of two independent measurements.



2D), demonstrating the efficacy of the generated H_2O_2 solution in water treatment. A maximal processing rate of $0.88 \text{ liter hour}^{-1}$ ($0.22 \text{ liter cm}^{-2} \text{ electrode hour}^{-1}$ or $2200 \text{ liters m}^2 \text{ electrode hour}^{-1}$) was achieved in lowering the TOC level to meet the drinking water standards, making our design economically and environmentally appealing for practical rainwater treatment when scaled up.

We also demonstrated that the oxidation reaction on the anode side could be flexibly modified to be coupled with the cathodic 2e^- -ORR for applications where H_2 is not available (fig. S14). Water oxidation to O_2 with concurrent proton release might be easier to access than hydrogen oxidation. A 0.5 M aqueous sulfuric acid solution on the anode side was used to lower the ionic resistance; H_2SO_4 was not consumed during the reaction and was continuously circulated (see materials and methods). The CEM membrane blocked crossover of the H_2SO_4 into the porous solid electrolyte layer, ensuring the formation of pure H_2O_2 solutions. This was confirmed by pH and ICP-OES measurements: The pH of the generated H_2O_2 solution was ~ 6 to 7 (pure H_2O_2 solutions show weak acidity), and the sulfur impurity level was $<10 \text{ ppm}$. High H_2O_2 productivity of $3.3 \text{ mmol cm}^{-2} \text{ hour}^{-1}$ ($3565 \text{ mol kg}_{\text{cat}}^{-1} \text{ hour}^{-1}$) could be achieved at a cell voltage of 2.13 V (Fig. 3A), representing an electricity-to-chemical energy conversion efficiency of 22.6% . H_2O_2 selectivity was very close to that observed with the $\text{O}_2//\text{SE}/\text{H}_2$ design at comparable current density (Fig. 2B), ruling out any impact on the cathodic 2e^- -ORR reaction by the anodic water oxidation. The ultrahigh purity of the synthesized H_2O_2 solution was confirmed using ICP-OES. A 100-hour test continuously generated pure H_2O_2 solutions, confirming the robust stability of the $\text{O}_2//\text{SE}/\text{H}_2\text{O}$ cell (fig. S15). To further simplify our process, we directly pumped air rather than purified O_2 to the cathode side (Fig. 3C). Although higher cell voltages were required to drive the reaction owing to substantially decreased O_2 concentration and activity, the air//SE// H_2O cell continued to provide H_2O_2 selectivity of $>90\%$. A maximal H_2O_2 partial current of $\sim 123 \text{ mA cm}^{-2}$ was reached at 2.71 V , corresponding to an impressive H_2O_2 productivity of $2.3 \text{ mmol cm}^{-2} \text{ hour}^{-1}$ ($2490 \text{ mol kg}_{\text{cat}}^{-1} \text{ hour}^{-1}$). To validate the scalability of our porous solid electrolyte design, we extended the electrode area from 4 cm^2

used for performance evaluation to $\sim 80 \text{ cm}^2$ in one unit modular cell (Fig. 3, D to F); these could be further stacked in the future for greater capacity. A maximal cell current of $>20 \text{ A}$ was achieved, with a high H_2O_2 selectivity of $\sim 80\%$ and a production rate of $\sim 0.3 \text{ mol hour}^{-1}$. Under a fixed cell current of 8 A , our scaled-up device produced highly concentrated pure H_2O_2 solutions of up to $20 \text{ wt } \%$ under a DI flow rate of 5.4 ml hour^{-1} (Fig. 3F and fig. S12B).

Given the wide variety of liquid products amenable to electrochemical synthesis, our solid electrolyte design could in principle be extended beyond H_2O_2 generation to other important electrochemical applications.

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SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/366/6462/226/suppl/DC1
Materials and Methods
Figs. S1 to S15
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Supplementary Text
References (54–67)

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RADIO ASTRONOMY

The low density and magnetization of a massive galaxy halo exposed by a fast radio burst

J. Xavier Prochaska^{1,2*}, Jean-Pierre Macquart³, Matthew McQuinn⁴, Sunil Simha¹, Ryan M. Shannon⁵, Cherie K. Day^{5,6}, Lachlan Marnoch^{6,7}, Stuart Ryder⁷, Adam Deller⁵, Keith W. Bannister⁶, Shivani Bhandari⁶, Rongmon Bordoloi⁸, John Bunton⁶, Hyerin Cho⁹, Chris Flynn⁵, Elizabeth K. Mahony⁶, Chris Phillips⁶, Hao Qiu¹⁰, Nicolas Tejos¹¹

Present-day galaxies are surrounded by cool and enriched halo gas extending for hundreds of kiloparsecs. This halo gas is thought to be the dominant reservoir of material available to fuel future star formation, but direct constraints on its mass and physical properties have been difficult to obtain. We report the detection of a fast radio burst (FRB 181112), localized with arcsecond precision, that passes through the halo of a foreground galaxy. Analysis of the burst shows that the halo gas has low net magnetization and turbulence. Our results imply predominantly diffuse gas in massive galactic halos, even those hosting active supermassive black holes, contrary to some previous results.

The low-density gas located in the outskirts of galaxies influences the process of galaxy formation, especially gas accretion and feedback (1). Absorption-line spectroscopy can detect this nearly invisible medium. Surveys demonstrate a very high incidence of cool gas (with temperature $T \sim 10^4$ K), detected through hydrogen Lyman series and continuum absorption, surrounding galaxies with masses similar to that of our Milky Way (1, 2). Properties of this gas depend on galaxy mass but are otherwise insensitive to the galaxy's internal properties (1, 3–5). Estimates for the total mass of the cool gas match or exceed the baryonic mass of the galaxy (4, 6). Theoretical treatments of halo gas around present-day galaxies disagree on the proportion of total mass retained in the halo during galaxy formation, with estimates ranging from several tens of percent up to all of the baryons predicted to accrete into the halo (7, 8). This uncertainty stems from observational insensitivity to the hot ($T \gtrsim 10^6$ K) gas that pervades galaxy halos (and within which the cold gas is embedded) and from systematic uncertainties in estimating its mass (1, 6). Constraints on the density and temperature of the halo gas are sufficiently limited to allow qualitatively different descriptions of its ionization and distribution (9, 10). The origin of the cool gas and its composition are challenging to explain theoretically; some models require cosmic rays and magnetic fields to transport material from the central galaxy to sustain the cool medium (11).

At coordinated universal time 17:31:15.48365 on 12 November 2018, the Commensal Real-

time ASKAP Fast Transients (CRAFT) survey on the Australian Square Kilometer Array Pathfinder (ASKAP) detected a fast radio burst (FRB 181112) from the 12 antennas active at the time. The burst arrival time swept across the observing band (≈ 1.129 to 1.465 GHz) (Fig. 1A), owing to propagation of the burst through a foreground plasma. The burst sweep yields an estimate of the FRB dispersion measure $DM_{\text{FRB}} = 589.27 \pm 0.03$ pc cm⁻³, which is the integrated density of electrons n_e at distance r from Earth scaled by $(1+z)^{-1}$, with z the redshift: $DM_{\text{FRB}} \equiv \int n_e / (1+z) dr$. The real-time detection triggered full download of the voltage data; these precisely localized the burst to a sky position $21^{\text{h}}49^{\text{m}}23.630^{\text{s}}$, $-52^{\circ}58'15.39''$ (right ascension, declination, J2000 equinox) with a statistical (systematic) error ellipse oriented at 120° east of north on the sky with major axis $a = 0.555''(3.2'')$ and minor axis $b = 0.153''(0.8'')$ (12).

Figure 1B shows a g -band image centered on FRB 181112, obtained with the FOcal Reducer/low dispersion Spectrograph 2 (FORS2) instrument on the Very Large Telescope (VLT). It shows the presence of a galaxy coincident with FRB 181112, previously cataloged by the Dark Energy Survey (DES) (13) as DES J214923.66–525815.28. The DES and FORS2 data also show a luminous galaxy $\approx 5''$ to the north of the FRB event (DES J214923.89–525810.43). We used follow-up spectroscopy with the FORS2 instrument to measure the redshift (12) of the former galaxy as $z = 0.47550$ and that of the latter galaxy as $z = 0.3674$ —i.e., in the foreground. We associate FRB 181112 with DES J214923.66–525815.28 (12). Compared to the other three known host galaxies of FRBs, the host

galaxy of FRB 181112 has an intermediate stellar mass of $M_\star \approx 10^{9.4}$ solar masses ($M_\odot$) (fig. S3) (12). It has colors matching star-forming galaxies at $z \sim 0.4$, has an estimated star formation rate of $0.6 M_\odot \text{ year}^{-1}$, and shows no signatures of an active galactic nucleus (AGN) (12).

The FRB sight line passes at an impact parameter $R_\perp = 29$ kpc from DES J214923.89–525810.43 (hereafter referred to as FG-181112), allowing us to probe the halo of this foreground galaxy. We analyzed the DES, FORS2, and complementary longer-wavelength Wide-field Infrared Survey Explorer (WISE) data to determine FG-181112's physical properties (12). We derive a high-stellar mass $\log_{10} M_\star / M_\odot = 10.69^{+0.22}_{-0.46}$, detect nebular emission lines indicative of an AGN and classify it as a Seyfert galaxy, and infer an old (age > 1.4 billion years) quiescent stellar population (Table 1 and table S5). Surveys of the halo gas surrounding galaxies of similar mass, with or without AGN activity (14), almost ubiquitously reveal strong absorption by cool ($T \sim 10^4$ K) gas for sight lines $R_\perp \leq 100$ kpc. Generally, the inferred total column densities of ionized gas exceed 10^{20} cm^{-2} (4, 6), and transitions of heavy elements indicate a turbulent velocity field (15), suggesting that a fraction of the gas has a relatively high density ($n_{\text{H}} \sim 1 \text{ cm}^{-3}$) (16). Such a foreground medium should influence the FRB signal.

The column of gas close to this massive galaxy, however, does not dominate DM_{FRB} . It contributes only $DM_{\text{FG}} \sim 50$ to 120 pc cm^{-3} , depending on assumptions for the density profile and total mass of the halo gas (12). The measured DM_{FRB} is consistent with models that include cosmic gas, our Galaxy, and the host (fig. S9) (17, 18). The sight line to FRB 181112 also intersects the edge of the Fermi Bubbles (12), a complex of hot gas encompassing the Galactic Center. The expected DM contribution from gas in these bubbles is small (12), but their entrained magnetic field may contribute to the FRB rotation measure (RM).

The RM is the density-weighted integral of the magnetic field parallel to the FRB sight line. The voltages recorded from the ASKAP antennas measure the electric field at the antenna locations in two orthogonal directions on the plane of the sky, enabling the linear polarization fraction of the burst radiation (and its position angle) to be measured as a function of frequency. Averaged over its duration, we find the burst to be $\sim 90\%$ linearly polarized and 10% circularly polarized (12). This can be used to estimate the burst RM,

¹University of California Observatories–Lick Observatory, University of California, Santa Cruz, CA 95064, USA. ²Kavli Institute for the Physics and Mathematics of the Universe, 5-1-5 Kashiwanoha, Kashiwa, 277-8583, Japan. ³International Centre for Radio Astronomy Research, Curtin University, Bentley, WA 6102, Australia. ⁴Department of Astronomy, University of Washington, Seattle, WA 98195, USA. ⁵Centre for Astrophysics and Supercomputing, Swinburne University of Technology, Hawthorn, VIC 3122, Australia. ⁶Commonwealth Science and Industrial Research Organisation, Australia Telescope National Facility, P.O. Box 76, Epping, NSW 1710, Australia. ⁷Department of Physics and Astronomy, Macquarie University, North Ryde, NSW 2109, Australia. ⁸Department of Physics, North Carolina State University, Raleigh, NC 27695, USA. ⁹School of Physics and Chemistry, Gwangju Institute of Science and Technology, Gwangju 61005, Korea. ¹⁰Sydney Institute for Astronomy, School of Physics, University of Sydney, Sydney, NSW 2006, Australia. ¹¹Instituto de Física, Pontificia Universidad Católica de Valparaíso, Casilla 4059, Valparaíso, Chile.

*Corresponding author. Email: xavier@uclick.org

Fig. 1. Dynamic spectrum of FRB 181112 and optical imaging of its host and a coincident foreground galaxy. (A) Dynamic spectrum of FRB 181112 recorded by ASKAP. The dispersion measure $DM_{\text{FRB}} = 589.27 \text{ pc cm}^{-3}$. $1 \text{ Jy} = 10^{-26} \text{ W m}^{-2} \text{ Hz}^{-1}$. (B) *g*-band FORS2 image centered on FRB 181112, the position of which is depicted by the red ellipses with solid and dashed lines indicating the statistical and systematic uncertainty, respectively. We estimate an additional systematic uncertainty of $\approx 0.5''$ in the astrometric solution of the FORS2 image. The host is well localized to a faint galaxy cataloged as DES J214923.66–525815.28, and one identifies a brighter galaxy (cataloged as DES J214923.89–525810.43, referred to as FG-181112) located $\approx 5.0''$ away at a PA $\approx 13^\circ$. The sight line to FRB 181112 passes through the halo of this foreground galaxy at an impact parameter $R_\perp = 29 \text{ kpc}$.

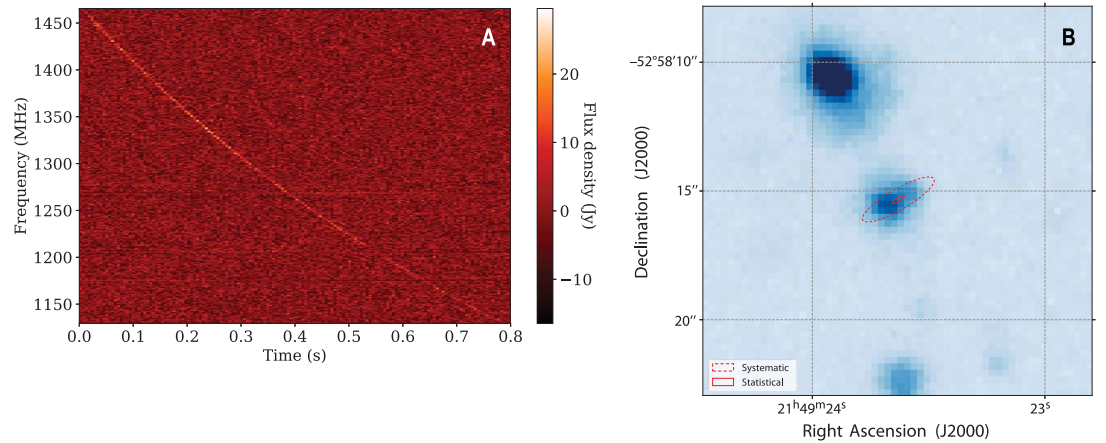
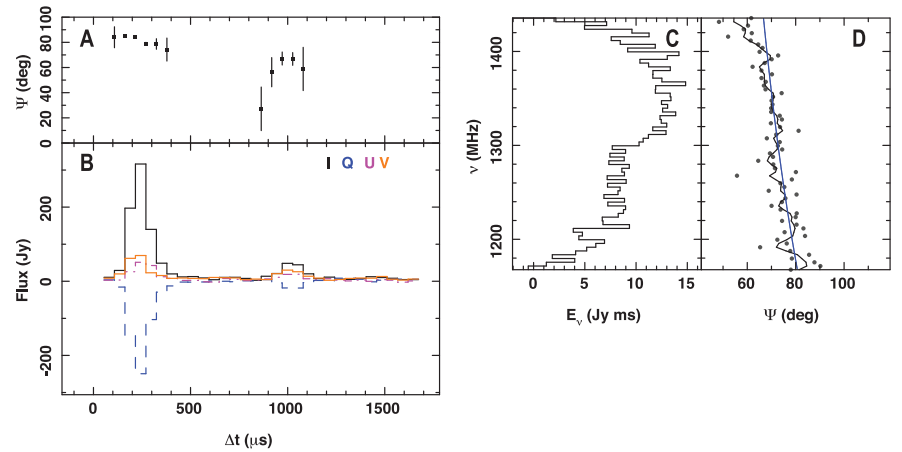


Fig. 2. Spectropolarimetric properties of FRB 181112.

(A) Relative linear polarization position angle Ψ of the burst averaged in frequency. Error bars indicate uncertainties in the measurements. (B) Polarimetric pulse profile of burst in four Stokes components (I, black solid line; Q, blue dashed line; U, purple dashed-dotted line; V, orange solid line). The two pulses, separated by $\sim 800 \mu\text{s}$, show different position angles. (C) Spectrum of fluence E_v , burst averaged over both pulses. (D) Position angle Ψ of the burst plotted as a function of frequency. The gray data points represent measurements in individual frequency channels; the black line denotes these measurements smoothed using a Gaussian kernel with SD = 4 MHz. The variation of the position angle with frequency is the result of Faraday rotation. The blue line shows a maximum likelihood model for polarization, using the inferred RM = 10.9 rad m^{-2} .



as $PA_{\text{obs}} = PA_{\text{int}} + (c/\nu)^2 \text{RM}$, where ν is the frequency, c is the speed of light, and PA_{obs} and PA_{int} are the observed and intrinsic polarization angles, respectively. Figure 2 depicts the frequency sweep of the polarization angle; the apparent ν^{-2} frequency dependence is the RM signature. We fitted an RM model to the sweep, yielding $\text{RM} = 10.9 \pm 0.9 \text{ rad m}^{-2}$. This is a low RM value, consistent (within the uncertainty) with the estimated RM due to our Galaxy toward FRB 181112 (12). Adopting an upper limit of $\text{RM} < 11 \text{ rad m}^{-2}$, we calculate an upper limit for the maximum parallel magnetic field $B_{\parallel}^{\text{max}}$ in the halo of FG-181112: $B_{\parallel}^{\text{max}} < 0.8 \mu\text{G} (n_e/10^{-3} \text{ cm}^{-3})^{-1} (\Delta L/30 \text{ kpc})^{-1}$, in the limit of a perfectly ordered magnetic field with ΔL a characteristic length scale through the halo. We have adopted fiducial values for n_e and L that may characterize the halo of FG-181112 (similar to those adopted for the DM_{FG} estimation). Field reversals would lead us to underestimate $B_{\parallel}^{\text{max}}$. Nevertheless, this low value for $B_{\parallel}^{\text{max}}$ implies that either the magnetic field in the halo is low compared

with the interstellar medium or that it is largely disordered.

These constraints have implications for the circumgalactic gas. The magnetic field value in equipartition with the thermal energy of the virialized halo gas is $B_{\text{eq}} \equiv \sqrt{8\pi n_e k_B T} = 2 \mu\text{G} (n_e/10^{-3} \text{ cm}^{-3})^{1/2} (T/10^6 \text{ K})^{1/2}$ with k_B the Boltzmann constant. Our $B_{\parallel}^{\text{max}}$ limit is similar to B_{eq} for physically motivated n_e , ΔL , and T , constraining the magnetic field to be near or below equipartition if the total field is similar to the net parallel field. Magnetic fields around the equipartition value enhance the rate of condensation of the hot circumgalactic gas into cooler clouds (11), as well as the survival of cool accreting gas (19). Near-equipartition field strengths are generated in some models in which cosmic ray pressure transports cool gas and metals to large distances from galaxies (20, 21). Our limit on $B_{\parallel}^{\text{max}}$ is below the mean estimate for sight lines that show strong gas absorption (22), despite our sight line likely intersecting gas with similarly strong absorption in FG-181112 (3).

The halo gas of FG-181112 broadens the width of the pulse at any given frequency. This temporal broadening τ_{scatt} arises from density fluctuations within the medium, which impose small differences in light-travel time for rays propagating through the gas (17, 23). This scattering is geometrical, and its effects are maximal for a scattering “screen” located at half the distance to the FRB. We determine an upper limit of $\tau_{\text{scatt}} < 40 \mu\text{s}$ due to scattering, constraining both the turbulent properties of the halo gas and its density. A 3-ms-wide pulse, 150 times the width of the FRB pulse, would still have been detected—i.e., the very narrow width of the FRB 181112 pulse is not the result of observational bias. Figure 2B shows that the temporal profile of FRB 181112 consists of two pulses separated by $\sim 800 \mu\text{s}$. The broadening limit is derived by modeling each component as a symmetric intrinsic pulse convolved with the one-sided exponential decay expected as a result of scattering (12). Temporal smearing due to inhomogeneities in the plasma distribution along

the line of sight would otherwise broaden the pulse to a frequency-dependent duration $\tau_{\text{scatt}}(\nu) = \tau_0(\nu/1 \text{ GHz})^\gamma$, where the index γ is typically ~ -4 (12).

The observed τ_{scatt} constrains the integral of the square of the density along the sight line, $\int dx \delta n_e(x)^2$, which we relate to the electron column density with the parameterization $\langle n_e \rangle \Delta L^{1/2} = \alpha^{-1} (\int dx \delta n_e^2)^{1/2}$, which takes the halo of FG-181112 to have characteristic length ΔL with an average density of $\langle n_e \rangle$. Thus, the parameter α encapsulates the root mean square amplitude of density fluctuations and the volume filling fraction of the turbulence, f_V . The limit on the in situ density assuming a Kolmogorov spectrum of turbulence (12) is

$$\langle n_e \rangle < 2 \times 10^{-3} \alpha^{-1} \left(\frac{\Delta L}{50 \text{ kpc}} \right)^{-1/2} \times \left(\frac{L_0}{1 \text{ kpc}} \right)^{1/3} \left(\frac{\tau_{\text{scatt}}}{40 \mu\text{s}} \right)^{5/12} \text{ cm}^{-3} \quad (1)$$

where $\Delta L \sim 50 \text{ kpc}$ approximates the path length through the foreground halo and L_0 is the outer scale of turbulence. As the turbulence is likely to be produced by galactic winds and inflows, we expect it to be driven at scales smaller than the impact parameter ($\sim 30 \text{ kpc}$) and consider $L_0 = 1 \text{ kpc}$ a reasonable value.

We now examine two standard models for halo gas in which the medium is composed of either hot ($T \sim 10^6 \text{ K}$) virialized gas or cool gas pressure-confined by the hot gas. In the case of hot virialized gas, our constraint on $\langle n_e \rangle$ suggests densities lower than those expected of $\sim 10^{-3} \text{ cm}^{-3}$ gas with kiloparsec driving scales (fig. S12). Because we expect the volume filling factor of this gas to be near unity, the upper limit on the density can be ameliorated only if the gas is much less turbulent (i.e., $\alpha \ll 1$) relative to galactic astrophysical plasmas, especially the interstellar medium of our Galaxy, where $\alpha \sim 7$ (12, 24).

For turbulent, cool 10^4 K clouds embedded in a hot medium, the constraints are stronger. Assuming pressure equilibrium with characteristic values for the hot gas $n_e = 10^{-3} \text{ cm}^{-3}$ and $T = 2 \times 10^6 \text{ K}$, application of Eq. 1 with $L_0 = 1 \text{ kpc}$ and $\Delta L = 50 \text{ kpc}$ yields $\alpha < 0.01$. Because $\alpha \propto f_V^{1/2}$, we require a filling factor of cool clouds of $f_V < 10^{-4}$ if the clouds are fully turbulent. Even lower values are required to satisfy this condition if the driving for turbulence within cool clouds is instead at parsec scales, which may be physically motivated (25).

These limits on the halo gas density derived from the scattering analysis contradict prior inferences that cool halo gas has a volume filling fraction of $f_V \sim 10^{-3}$ to 10^{-2} (6, 26, 27). The total neutral hydrogen column density offers the most direct comparison to our result: Photoionization equilibrium constrains the same combination of parameters as scattering, implying

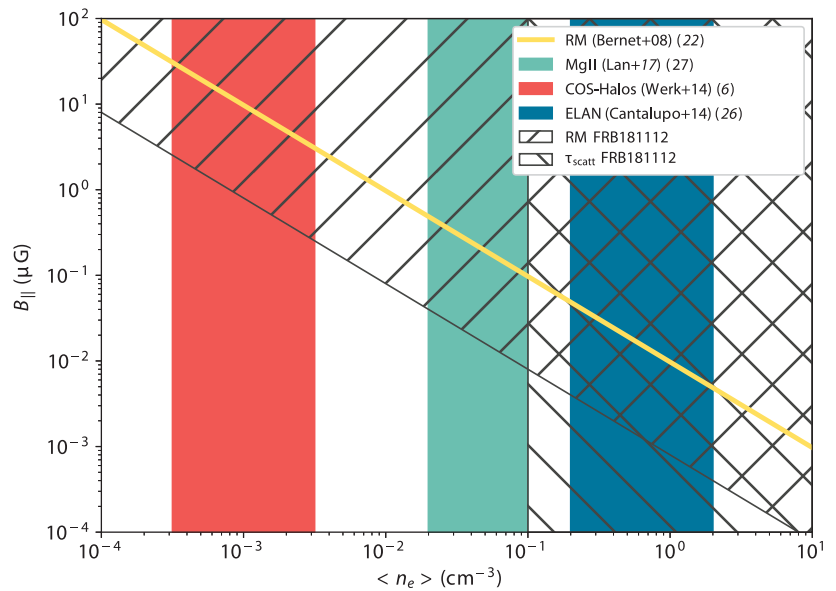


Fig. 3. Constraints on the coherent magnetic field parallel to the line of sight $B_{||}$ and electron density n_e in the halo of FG-181112. The hatched regions show the parameter space in $B_{||}, n_e$ (cool gas) ruled out for the halo of FG-181112 from the measured RM and τ_{scatt} of FRB 181112. These constraints are largely independent of the properties of the foreground galaxy. We compare these results with previous inferences for the density of cool halo gas (colored regions), based on ionization modeling and Ly α fluorescence. We also illustrate previous estimations for the magnetic field strength in halo gas (yellow line) (22), which conflict with our results.

Table 1. Properties of FRB 181112, its host, and the foreground galaxy FG-181112. The two uncertainties in right ascension and declination are statistical and systematic, projected onto the coordinate axes. These uncertainties are best described as ellipses with position angle 120° east of north and major and minor axes of $a_{\text{statistical}} = 0.55''$, $b_{\text{statistical}} = 0.15''$ and $a_{\text{systematic}} = 3.2''$, $b_{\text{systematic}} = 0.8''$. The coherent magnetic field, density, and filling factor estimates assume a characteristic path length through the halo of $\Delta L = 50 \text{ kpc}$. The density and filling factor estimates assume a driving scale with root mean density fluctuations of 1 at $L_0 = 1 \text{ kpc}$, with the bound scaling as $\propto L_0^{1/3}$, as well as a Kolmogorov spectrum of turbulence to separations below the diffractive scale r_{diff} . The filling factor estimate further assumes that cool $T_{\text{cool}} = 10^4 \text{ K}$ gas is in pressure equilibrium with hot gas with density $\langle n_e \rangle = 10^{-3} \text{ cm}^{-3}$ and temperature $T_{\text{hot}} = 2 \times 10^6 \text{ K}$ hot gas, with the bound scaling as $\propto (\langle n_e \rangle T_{\text{hot}} / T_{\text{cool}})^{-2}$. See text and (12) for details.

Property	Value
<i>FRB</i>	
Right ascension (J2000)	$327.34846^\circ \pm 0.00007^\circ \pm 0.0006^\circ$
Declination (J2000)	$-52.97093^\circ \pm 0.00004^\circ \pm 0.0002^\circ$
Dispersion measure (DM_{FRB})	$589.27 \pm 0.03 \text{ pc cm}^{-3}$
Rotation measure (RM)	$10.9 \pm 0.9 \text{ rad m}^{-2}$
Pulse width	$< 40 \mu\text{s}$
<i>Host galaxy</i>	
Redshift	0.47550 ± 0.00015
Stellar mass	$2.6 \pm 1.1 \times 10^9 M_\odot$
Star formation rate	$0.6 M_\odot \text{ year}^{-1}$
<i>Foreground galaxy FG-181112</i>	
Redshift	0.36738 ± 0.00007
Impact parameter to the FRB sight line ($R_\perp$)	$29 \pm 3 \text{ kpc}$
Stellar mass	$4.9 \pm 3.2 \times 10^{10} M_\odot$
Star formation rate	$< 0.3 M_\odot \text{ year}^{-1}$
Coherent magnetic field parallel to the line of sight	$B_{ } < 0.5 \mu\text{G} (n_e / 10^{-3} \text{ cm}^{-3})$
Density constraint for hot, diffuse gas ($f_V \sim 1$)	$n_e < 2 \times 10^{-3} \text{ cm}^{-3}$
Filling factor constraint for cool, clumpy gas	$f_V < 10^{-4}$

$(n_e/0.1 \text{ cm}^{-3})(f_V/10^{-3})^{1/2}(\Delta L/50 \text{ kpc})^{1/2} \sim 1$ if we take a typical neutral hydrogen column density of 10^{18} cm^{-2} at 30 kpc measured for halos with similar masses as FG-181112 (4). Reconciling these values with the scattering from FG-181112 either implies that the cool clouds are less turbulent than assumed or that our sight line has less cool gas than is typical. The foreground galaxy is classified as a Seyfert, with an embedded accreting supermassive black hole in a central AGN that could lead to a more evacuated halo (28), although it has been argued that such activity may lead to more cool gas (29). Even if the clouds are not turbulent and instead we consider the refractive bending of light through a network of parsec-scale clouds (25), we rule out a population of 0.1-pc (or smaller) clouds with $f_V \sim 10^{-3}$ (12).

FRBs experience a number of propagation effects that render them sensitive probes of the density, magnetic field, and turbulence of the otherwise elusive gas that pervades galaxy halos. The constraints derived from FRB 181112 for the halo of a massive galaxy are summarized in Fig. 3. The $n_e, B_{\parallel}$ parameter space ruled out by our observations conflicts with several previous inferences for halo gas (22, 26, 27). Our observations indicate a density of hot gas that is lower than in many models and also a column of cool gas that is smaller than commonly inferred.

Our results demonstrate that FRBs can be used to elucidate the physical properties of diffuse gas in the halos of galaxies. The multiple pulses observed in FRB 181112 could be due to multipath propagation through the gas. That would be a natural consequence of a medium comprising very low filling factor cool clouds embedded in hot virialized halo gas, with the pulse multiplicity signifying the number of clouds intersected and their arrival times yielding their offsets from the direct burst sight line.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Supplementary Text
Figs. S1 to S12
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SURFACE CHEMISTRY

Chemical bond formation showing a transition from physisorption to chemisorption

Ferdinand Huber¹, Julian Berwanger¹, Svitlana Polesya², Sergiy Mankovsky², Hubert Ebert², Franz J. Giessibl^{1*}

Surface molecules can transition from physisorption through weak van der Waals forces to a strongly bound chemisorption state by overcoming an energy barrier. We show that a carbon monoxide (CO) molecule adsorbed to the tip of an atomic force microscope enables a controlled observation of bond formation, including its potential transition from physisorption to chemisorption. During imaging of copper (Cu) and iron (Fe) adatoms on a Cu(111) surface, the CO was not chemically inert but transitioned through a physisorbed local energy minimum into a chemisorbed global minimum, and an energy barrier was seen for the Fe adatom. Density functional theory reveals that the transition occurs through a hybridization of the electronic states of the CO molecule mainly with s -, p_z -, and d_z^2 -type states of the Fe and Cu adatoms, leading to chemical bonding.

The physicist Richard Feynman believed that the sentence “...all things are made of atoms—little particles that move around in perpetual motion, attracting each other when they are a little distance apart, but repelling upon being squeezed into one another” (1) contains the most information about scientific knowledge in the fewest words. Although this quote captures the key characteristics of chemical bonding, subtle complications do occur in nature. In 1932, Lennard-Jones described that molecules can bond to a surface in two ways [see figure 3 in (2)]: a weak bond induced by van der Waals (vdW) attraction (physisorption) and, for smaller distances, a stronger chemical bond (chemisorption). In some cases, these two bonding regimes are split by an energetic barrier and, depending on the height of the barrier, transitions can occur [see figure 9.5 in (3)]. Overall, three different bonding scenarios can evolve (3, 4):

1) The formation of a weak physical bond (vdW bond) with depth of ≈ 20 meV (0.46 kcal/mol) as shown by the potential energy V versus distance z curve in Fig. 1A and its corresponding force curve $F_z(z)$ in Fig. 1D with a maximal attractive force (5) on the order of 10 pN. The interaction of two noble gas atoms such as Xe is an example of such an interaction.

2) The formation of a strong chemical bond with energies on the order of electron volts shown in Fig. 1B, where the attractive force (Fig. 1E) can reach nanonewtons and mask the ever-present vdW forces that are on the order of 10 pN, followed by repulsion at small z . The data in Fig. 1, B and E, correspond to the bonding energy and vertical force between

two Si atoms according to the Stillinger-Weber potential (6).

3) The third bonding mechanism involves a transition from physisorption to chemisorption as shown in Fig. 1C (3, 4). The initial appearance of a weak vdW bond is followed by a transition that can show an energy barrier of high strength (black curve in Fig. 1C), a medium barrier (green and red curves), and a vanishing barrier (blue curve). If a molecule arrives at the surface with sufficient thermal energy to overcome the slight energy barrier of the green energy curve in Fig. 1C, it can chemisorb immediately. If a stronger energy

barrier occurs, as shown by the black curve in Fig. 1C, its energy needs to be lifted by thermal excitation to overcome the barrier and to form a strong chemical bond (Fig. 1F). The $V(z)$ curve in Fig. 1C is key to the physisorption-chemisorption transition and possible subsequent heterogeneous catalysis. Whereas previous methods only provided the equilibrium positions at their corresponding temperatures, state-of-the-art atomic force microscopy (AFM) at low temperatures can directly record this curve.

Carbon monoxide can undergo physisorption as well as molecular and dissociative chemisorption on transition metal surfaces. Dissociative chemisorption to adsorbed C and O atoms tends to prevail on all transition metals in the periodic table left of a boundary between iron and cobalt at room temperature [chapter 6.4.1 in (4), and (7, 8)], as well as for W (9). Conventional methods for adsorption studies, such as thermal desorption spectroscopy or electron energy loss spectroscopy (3, 4), probe large molecular ensembles. Chemisorption is key to heterogeneous catalysis, and detailed knowledge about its basic mechanism can be obtained by using scanning tunneling microscopy (STM) as an atomic probe (10). Although STM combined with ultrashort laser pulsing has recently obtained femtosecond time resolution in imaging the surface vibrations of molecules (11), STM has thus far been used to image the end products of surface reactions and not the reactions themselves.

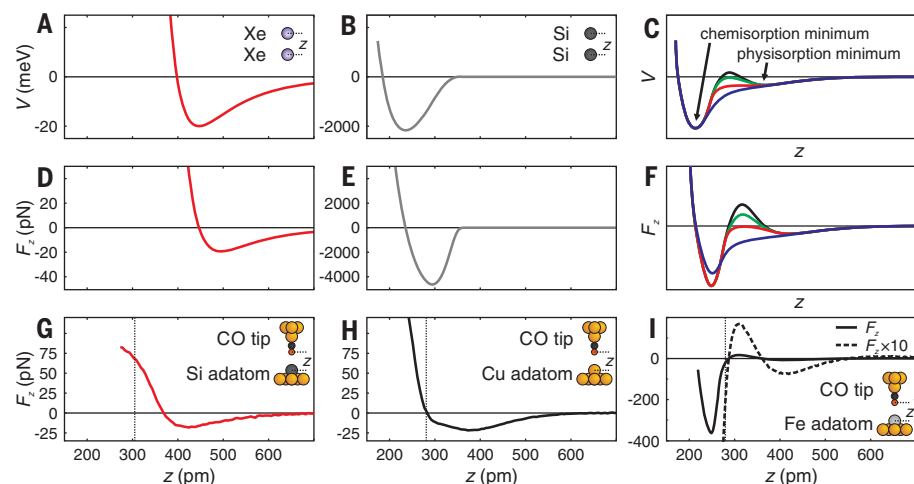


Fig. 1. Force and energy versus distance curves for various bonding situations. (A to C) Schematic potential energy V and (D to F) vertical force F_z versus distance z curves for [(A) and (D)] a weak physical bond, [(B) and (E)] a strong chemical bond, and [(C) and (F)] a bond with a transition from physisorption to chemisorption. The colored curves in (C) and (F) show four different cases that vary by their barrier height. If a repulsive energy barrier exists (i.e., $V > 0$ in the region between physisorption and chemisorption as shown with a black curve), the adsorbate may just reach the physisorbed state. For a very low energy barrier (green curve), thermal excitation can suffice to bring the adsorbate into a chemisorbed state, and for the red and blue curves, the adsorbate will immediately end up in the chemisorbed state. (G to I) Experimental force versus distance curves showing various bonds between the CO-terminated tip and (G) a Si adatom (24), (H) a Cu adatom, and (I) a Fe adatom on Cu(111). The potential energy curve corresponding to (I) is shown in fig. S10.

¹Institute of Experimental and Applied Physics, Department of Physics, University of Regensburg, 93040 Regensburg, Germany. ²Department of Chemistry, Ludwig-Maximilians-Universität München, 81377 Munich, Germany.

*Corresponding author. Email: franz.giessibl@ur.de

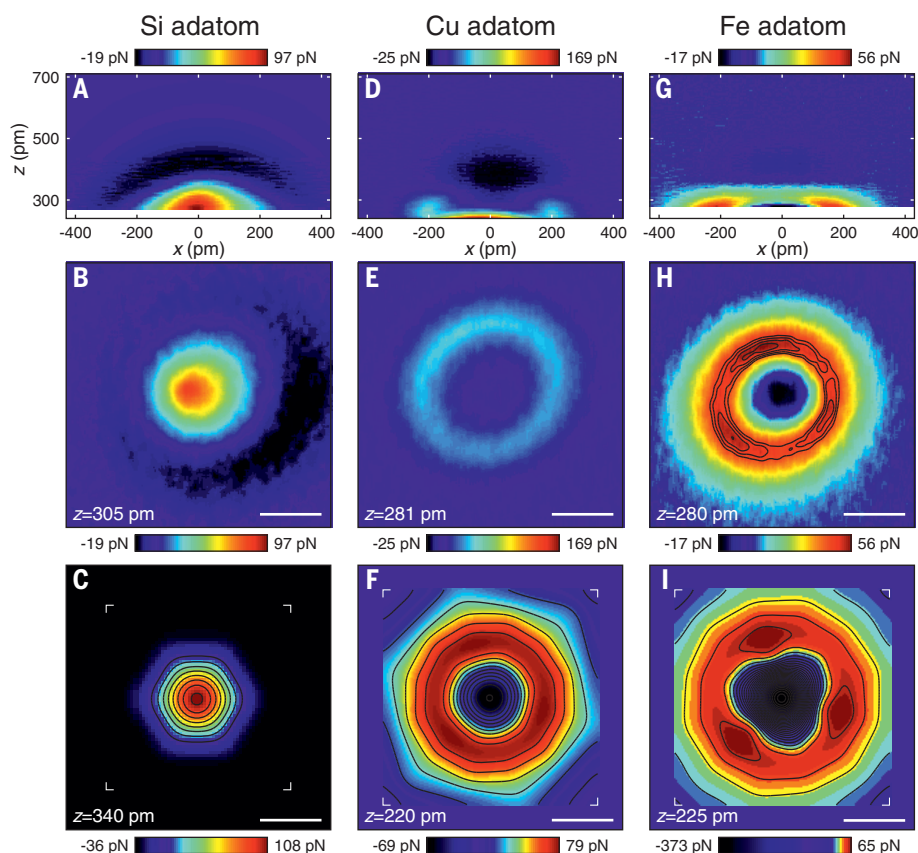


Fig. 2. Experimental and calculated forces for three different adatoms in side and top views. Top row (side view): Experimental vertical forces F_z in the xz plane between a CO-terminated AFM tip and (A) a Si adatom, (D) a Cu adatom, and (G) a Fe adatom on Cu(111). Middle row (top view): Constant-height force data in the xy plane between a CO-terminated tip and (B) a Si adatom, (E) a Cu adatom, and (H) a Fe adatom on Cu(111) taken at z positions, as indicated by vertical dotted lines in Fig. 1, G to I, respectively. Bottom row (top view): DFT calculations of F_z in the xy plane between a CO molecule tip and (C) a Si adatom, (F) a Cu adatom, and (I) a Fe adatom on Cu(111). The three local maxima on the experimental data (H) and DFT data (I) for the Fe adatom are located above the hollow sites of the Cu(111) substrate underneath (see figs. S5 and S6). Note that the color scale is the same for the force data in the top and middle rows. The color scale in the bottom row is different in order to maximize the contrast. Scale bars, 200 pm.

AFM (12) and its variants (13, 14) have become a powerful tool for surface studies (15). The attachment of a CO molecule to an STM tip can enhance resolution by creating a sharper probe tip (16), and Gross *et al.* reported that CO-terminated AFM tips allow imaging of organic molecules with intramolecular resolution (17), leading to wide use of CO-terminated tips (18). The inertness of CO-terminated tips enabled the imaging of many organic molecules (18) and graphene (19), as well as metal clusters and the silicon (111)-(7×7) surface (20), at unprecedented resolution. The use of CO-terminated AFM tips allows the tracking of the formation and potential transition from physisorption to chemisorption of a bond as a function of distance (i.e., reaction coordinate) for a single CO molecule with a precisely controlled position on a picometer scale.

There is a restriction imposed by the bond of the CO molecule to the tip. A CO molecule

in the gas phase can orient itself freely on a surface to enable the maximal bonding strength. In metal carbonyls such as $\text{Ni}(\text{CO})_4$ or $\text{Fe}(\text{CO})_5$, CO bonds with the C atom to the transition metal (21) and CO bonds to the AFM's metal tip in a similar manner. Experimental and theoretical evidence holds that the oxygen end of the CO-terminated tip is chemically inert. When imaging pentacene with CO-terminated tips (17), density functional theory (DFT) has shown that Pauli repulsion between electrons provides the contrast (22, 23).

The bottom row in Fig. 1 displays experimental $F_z(z)$ curves over the centers of three different adatoms obtained with CO-terminated tips. Figure 1G shows the interaction of a CO-terminated tip with a single Si adatom on Cu(111), as indicated in the inset. The attractive vdW force reached only −20 pN before Pauli repulsion forces dominated (24). The interaction of the CO-terminated tip with the

Si adatom resembled physisorption—a weak attraction turns to Pauli repulsion with a single energetic minimum. The strong covalent bonds with a magnitude of nanonewtons shown in Fig. 1, B and E, were used to atomically resolve AFM images in vacuum on the silicon surface (25), where DFT identified a covalent character (26) that was verified by precise force spectroscopy (27, 28).

Figure 1H shows the $F_z(z)$ curve for a CO-terminated tip over a Cu adatom on Cu(111). The attractive force minimum was at $z = 373$ pm, and the attractive z range was widened compared with the Si atom in Fig. 1G. Figure 1I shows the $F_z(z)$ curve for a CO-terminated tip over a Fe adatom on Cu(111), which resembles the qualitative physisorption-chemisorption transition of Fig. 1F (black curve). The physisorbed force minimum of −8 pN at $z = 420$ pm was followed by a force barrier of +17 pN at $z = 310$ pm and a maximal attractive force of −364 pN at $z = 250$ pm. The occurrence of a barrier in the experimental force curve for the Fe adatom in Fig. 1I and its similarity to the schematic force curves pertaining to a physisorption-chemisorption transition in Fig. 1F pointed to the experimental observation of such a transition, as elucidated below.

The $F_z(z)$ curves in Fig. 1, G to I, were measured with the CO-terminated tip exactly centered over the adatoms. However, F is not merely a function of absolute distance between the centers of the O atom of the tip and the adatom, it is also a function of the polar and azimuthal angles with respect to the surface normal and substrate orientation. The top row of Fig. 2 shows F in the z direction as a function of lateral x direction and z position at $y = 0$. The force fields for the three different adatoms were distinctly different in the xz plane. The force curves in Fig. 1, G to I, are traces of the two-dimensional force fields $F_z(x, y, z)$ at $x = y = 0$. The middle row shows experimental constant-height force images of the three adatoms. The bottom row displays DFT force calculations for the three different adatoms.

The left column of Fig. 2 shows data for the simplest case, the Si adatom. For the force data of the Si adatom on the xz plane in Fig. 2A, we initially found weak vdW attraction followed by strong Pauli repulsion that was roughly proportional to the total charge density of the Si adatom as displayed in fig. S1A. The Si adatom appeared in the xy plane (Fig. 2B) as a Gaussian-shaped repulsion, showing that the CO-terminated tip interacted with it in a similar manner as it does with organic molecules.

Simulations of this image for four different heights using the probe particle model (29, 30) are shown in fig. S2, where the lateral bending of the CO-terminated tip (31) was taken into account. The DFT calculation of the force image (Fig. 2C) yielded a result similar to that of

the experimental data (32). Because Pauli repulsion was the contrast mechanism here, the experimental images resembled the total charge densities presented in fig. S1A. DFT confirmed that Pauli repulsion was the contrast mechanism—differential charge density plots and calculations of the energies of the states (see figs. S7, A to D, and S8, A to F) showed no evidence for chemical bonding.

For the Cu adatom data (middle column of Fig. 2), in the center at $x \approx 0$ in Fig. 2D, vdW attraction was followed by some more slight attraction before turning to Pauli repulsion. The circumference of the Cu adatom at $x \approx \pm 200$ pm looked completely different with a transition from vdW attraction directly to Pauli repulsion. Accordingly, the constant-height data in Fig. 2E shows a ringlike appearance. The DFT calculation in Fig. 2F resembles the experimental data in Fig. 2E and is vastly different from the total charge density of the Cu adatom shown in fig. S1B. The evolution of the contrast with distance starts from the attractive vdW signature, changes to the repulsive ring, and ends in a repulsive cusp in the center, as shown in detail in fig. S3. The calculated $F_z(z)$ curves (fig. S7E), differential charge density plots (fig. S7, F to H), and pronounced shifts in the energies of the electronic states (fig. S8, G to M) provided a consistent dataset indicating the emergence of a medium-strength bond (33). The physical origin for the delayed transition from vdW attraction to Pauli repulsion is a hybridization of the electronic states of the Cu adatom with the states of the CO-terminated tip (34).

For the Fe adatom (right column of Fig. 2), in the center at $x \approx 0$ in Fig. 2G, the interaction started with vdW attraction (dark lenticular area at $z \approx 400$ pm), followed by weak repulsion (light-green lenticular area at $z \approx 330$ pm). After penetrating the repulsive barrier in the center, attraction occurred (see also Fig. 1I). For even smaller z , we expected repulsion again, but this close distance is not accessible because approaching to such close distances risked the integrity of the CO-terminated tip (35). Outside the center, at $x \approx \pm 210$ pm, we saw a direct transition from vdW attraction to Pauli repulsion similar to the circumference of the Cu adatom. The top view at Fig. 2H shows a repulsive ring similar to the Cu adatom, but for the Fe adatom, three local maxima were located over the hollow sites of the underlying Cu(111) surface (see figs. S5 and S6). The DFT force calculations presented in Fig. 2I confirmed the presence of three local maxima on the repulsive ring in registry with the Cu(111) substrate (see fig. S6).

As in the case of the Cu adatom, the images of the Fe adatom did not relate to the total charge density of the Fe adatom shown in fig. S1C. The physical origin of the ringlike appearance and strong attraction in the center of the

Fe adatom was a hybridization of electronic states between tip and sample as revealed by the DFT calculations. The $F_z(z)$ curves (fig. S7I), differential charge density plots (fig. S7, J to L), and pronounced shifts in the energies of the electronic states of the CO-terminated tip and Fe adatom (fig. S8, N to T) provided a coherent picture of the formation of a chemical bond resulting from hybridization (33). We note that the appearance of the Cu and Fe adatoms as repulsive tori is not an artifact of bending of the CO-terminated tip (31) (see fig. S9).

The experimental images of Cu and Fe adatoms showed similarities and differences. Both appear as repulsive tori when imaged with CO-terminated tips at close distance. However, the Fe adatom showed three distinctive local maxima on the torus, and the attractive force in the center reached values down to -364 pN, whereas the center of the Cu adatom was much less attractive and even allowed to image the repulsive cusp for very small distances. Previous experiments have shown that single Fe adatoms on Cu(111) have a magnetic moment (36). Our DFT calculations confirm this and find zero magnetic moment for the Cu adatom. Thus, the physical origin of the difference in the AFM data of Cu versus Fe adatoms is the element-specific occupation of the majority and minority 3d spin states (see fig. S12).

We have shown that CO-terminated tips can hybridize with sample atoms and produce a contrast that is much different from the total charge density. The subatomic contrast (20), i.e., the appearance of nontrivial structures within images of single atoms, was explained as a signature of hybridization of states with a s, p, and d character in the formation of chemical bonds. The present findings extend atomically resolved force microscopy into a previously unexplored interaction regime. When atomically resolved AFM in vacuum was introduced 25 years ago, strong covalent or ionic bonds were probed in a noncontact distance regime, and noncontact AFM and atomically resolved AFM have, historically, often been considered synonymous. The introduction of CO-terminated tips by Gross *et al.* (17), as well as noble gas and other inert tips (37), expanded the distance regime where nondestructive atomically resolved images are possible from the noncontact regime to an intermittent-contact mode that probes Pauli repulsion forces.

The present work further expands AFM into a distance regime where the hybridizations occur that underlie the chemical bond. Possible applications include the study of partially unfilled Cu 3d states in cuprate superconductors (38). We showed that CO-terminated tips are not generally chemically inert, as tips terminated by noble gas atoms are. Therefore, CO-terminated tips do not generally interact

via Pauli repulsion with the total charge density of the sample. This might change the interpretation of images of organic molecules that contain metal ions, in particular those with unfilled 3d shells.

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and S8, as well as the experimental observation of the repulsive barrier above the Fe adatom, showed that the hybridization occurs only under the presence of the CO-terminated tip and it involves not only s and p states but also d states.

35. The CO-terminated tip can come quite close to the Fe adatom when located at the center of the Fe adatom where lateral forces are zero. In Fig. 1I, the tip was even approached almost to the equilibrium distance where the force is zero again after passing the maximal attraction of -364 pN at the distance of 250 pm. The minimal distance that can be sustained by the AFM tip without losing its CO termination is determined by experience. Usually, tip loss is imminent when the driving signal that controls the constant oscillation amplitude of the force sensor starts to rise, i.e., when damping of the sensor owing to the tip-sample interaction becomes noticeable. When scanning in the xy plane, lateral forces act on the CO-terminated tip and larger distances are required to prevent the loss of the CO termination (compare Fig. 2G where the minimal distance was almost 100 pm larger than in the force spectrum of Fig. 1I).

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(original draft). All authors reviewed and edited the manuscript.

Competing interests: F.J.G. holds patents for the force sensor that was used in the experiment. All other authors declare no competing interests. **Data and materials**

availability: All relevant data are available in the main text or the supplementary materials. All raw data and scripts that were used for the data analysis are stored in the computer center of the University of Regensburg and are available on request.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S12
References (39–50)

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SUPERCONDUCTIVITY

Observation of half-quantum flux in the unconventional superconductor β -Bi₂Pd

Yufan Li^{1*†}, Xiaoying Xu^{1*}, M.-H. Lee², M.-W. Chu², C. L. Chien^{1,3,4†}

Magnetic flux quantization is one of the defining properties of a superconductor. We report the observation of half-integer magnetic flux quantization in mesoscopic rings of superconducting β -Bi₂Pd thin films. The half-quantum fluxoid manifests itself as a π phase shift in the quantum oscillation of the superconducting critical temperature. This result verifies unconventional superconductivity of β -Bi₂Pd and is consistent with a spin-triplet pairing symmetry. Our findings may have implications for flux quantum bits in the context of quantum computing.

The condensation of Cooper pairs gives rise to superconductivity (1). A key signature of the electron pairing is the quantization of the magnetic flux through a multiply connected superconducting body, in discrete units of $\Phi_0 = hc/2e$, where h is the Planck constant, c is the speed of light, and e is the elementary charge. Indeed, the observations of the fluxoid quantization served as the first experimental verifications of the Bardeen–Cooper–Schrieffer theory of conventional superconductors (SCs) (2–4). Shortly after the initial magnetometry measurements, Little and Parks further demonstrated oscillations of the superconducting transition temperature T_c as a function of the applied magnetic flux resulting from the corresponding periodicity of the free energy of the superconducting state (5). The minimum of the free energy, or the maximum of the T_c , is achieved when the applied magnetic flux takes the form $\Phi = n\Phi_0$, where n is an integer number. In the following decades, the Little–Parks effect, as a stringent test for the electron pairing, has been observed in numerous superconducting materials (6–9). However, in spin-triplet SCs, half-quantum magnetic fluxes (HQFs) of $(n + 1/2)\Phi_0$ have been predicted by Geshkenbein, Larkin, and Barone (GLB) to be energetically more favorable than integer ones (10). The idea was later extended to the spin-singlet high- T_c SCs (11) and realized in YBa₂Cu₃O_{7- δ} tricrystals with delicately designed crystalline boundaries (12, 13), which, in conjunction with the corner junction experiments (14, 15), pinpointed the d-wave pairing symmetry. More recently, experimental indications of a different half-quantum vortex effect have been reported in the spin-triplet SC candidate

Sr₂RuO₄ (16, 17). This phenomenon manifests itself as a splitting of the integer-quantization steps under an additional in-plane magnetic field (18); it is not to be confused with the HQF effect proposed by GLB. The spin-triplet nature of Sr₂RuO₄ remains to be unequivocally concluded (19–22).

The past decade has witnessed a surging interest in topological superconductors (TSCs), which may host Majorana fermions (19, 20, 23). TSCs are considered to have a profound connection to the spin-triplet pairing (24–26). The search goes on in other superconducting materials, including doped topological insulators, noncentrosymmetric SCs (19, 20), and iron-based SCs (27).

Of particular interest is the material β -Bi₂Pd with a centrosymmetric tetragonal crystal structure (28), which is reported to host spin-polarized topological surface states that coexist with superconductivity (29, 30). One scanning tunneling spectroscopy study in particular reports observation of Majorana-bound states at the center of the vortices in epitaxial thin films (31). Controversy persists, however, because other tunneling spectroscopy and calorimetric studies in bulk specimens suggest the conventional s-wave pairing mechanism (32–34). To explore the nature of superconductivity in this material, we performed Little–Parks experiments on mesoscopic superconducting rings fabricated on textured β -Bi₂Pd thin films. We found that the flux quantization becomes $(n + 1/2)\Phi_0$. In other words, the oscillation of T_c as a function of the applied magnetic flux is shifted by a phase of π . This is the experimental signature of the HQF predicted by GLB (10). Our findings imply that the superconductivity of β -Bi₂Pd originates from an unconventional pairing symmetry consistent with spin-triplet pairing.

The fluxoid Φ' of a superconducting loop was introduced by F. London as $\Phi' = \Phi + (4\pi/c)\oint \lambda^2 \vec{j}_s \cdot d\vec{s}$ where Φ is the applied magnetic flux, λ is the London penetration depth, and $\vec{j}_s$ is the supercurrent density (35). Φ' must take quantized values, with integral increments of a flux quantum Φ_0 (2, 4, 35). The

applied magnetic flux Φ can take arbitrary values, which requires $\vec{j}_s$ to compensate it to maintain the quantized Φ' . This leads to periodic oscillations of the free energy, and in turn T_c , in response to the applied magnetic field (5). Usually, a circulating $\vec{j}_s$ is not required when the external field satisfies $\Phi = n\Phi_0$; for these values of the applied flux, the free energy of the superconducting state is the lowest and, as a result, the T_c is the highest. The maximum of the free energy and the minimum of T_c occur when $n - \Phi/\Phi_0 = \pm \frac{1}{2}$, as depicted in Fig. 1A. For unconventional SCs, the superconducting order parameter becomes anisotropic, retaining the symmetry of the underlying crystal lattices. In polycrystalline samples, it is possible, as suggested by GLB, for the phase factor of the complex order parameter to experience an additional phase shift of π along a path across the boundary of two crystal grains, resulting in a sign change in the corresponding free-energy term (10). An odd number of the π phase shifts accumulated around a superconducting loop will reverse minima and maxima of the total free energy. It is thus the maxima of T_c , instead of the minima, that occur when $n - \Phi/\Phi_0 = \pm \frac{1}{2}$, as shown in Fig. 1B; the fluxoid quantization becomes $\Phi' = (n + 1/2)\Phi_0$. In the absence of an external magnetic field, such a superconducting loop will hold an HQF as its ground state. GLB concluded that this is most likely to occur in polycrystalline p-wave SCs (10).

To examine the fluxoid quantization in β -Bi₂Pd, we fabricated submicrometer-sized ring devices using 50-nm-thick, (001)-textured β -Bi₂Pd thin films deposited on oxidized silicon substrates by magnetron sputtering (36). The size of the ring is important because the oscillation occurs in units of $\Phi_0 \approx 20$ Gauss- $(\mu\text{m})^2$. For a ring of 1 μm by 1 μm , oscillations occur in field increments of ~ 20 Oe. Measuring much larger rings is far more demanding on the field resolution and makes it more difficult to determine the zero-external-field state in the presence of the remnant field of the magnet. For smaller rings, the size becomes comparable to the coherence length. A representative device geometry can be found in Fig. 1C, with a mean size of 0.8 μm by 0.8 μm . Control samples with the same device geometries were patterned using 28-nm-thick thin films of Nb, which is a conventional s-wave SC. The temperature dependence of the device resistance is shown in Fig. 1D. A broadening of the transition width (~ 1.5 K) compared with that of the as-grown films (< 0.2 K) is typical for submicrometer-sized devices (7–9, 17, 37). The Little–Parks effect can be observed when the sample is placed at a fixed temperature within the superconducting transition regime, where the variation of the T_c manifests as oscillations of the

¹Department of Physics and Astronomy, Johns Hopkins University, Baltimore, MD 21218, USA. ²Center for Condensed Matter Sciences and Center of Atomic Initiative for New Materials, National Taiwan University, Taipei 10617, Taiwan. ³Institute of Physics, Academia Sinica, Taipei 11519, Taiwan. ⁴Department of Physics, National Taiwan University, Taipei 10617, Taiwan.

*These authors contributed equally to this work.

†Corresponding author. Email: yili171@jhu.edu (Y.L.); clchien@jhu.edu (C.L.C.)

Fig. 1. Little-Parks effect and the experimental setup. (A) Schematic drawing of the Little-Parks effect of conventional s-wave SCs. The critical temperature (upper panel) and the resistance (lower panel) oscillate as a function of the applied magnetic flux in the period of one magnetic flux quantum $\Phi_0 = hc/2e$. The fluxoid takes integer-quantized values of $\Phi' = n\Phi_0$. (B) Schematic drawing of the effect of the half-quantum fluxoid manifested as a π phase shift in the Little-Parks oscillation. The fluxoid takes fractionally quantized values of $\Phi' = (n + 1/2)\Phi_0$. (C) Top: scanning electron microscope image of a representative superconducting ring device. Middle: schematic drawing of the experimental setup. Bottom: schematic drawing of the cross section of the ring structure. The dimensions are not drawn to scale. (D) Temperature dependence of the resistance of the β -Bi₂Pd and Nb ring devices. The film thicknesses of β -Bi₂Pd and Nb are 50 and 28 nm, respectively. (E) Little-Parks effect of the Nb ring device showing the ordinary case depicted in (A). The resistance oscillates as a function of the perpendicular magnetic field. The sample was held at a constant temperature of 5.7 K in the vicinity of the normal-superconducting phase transition.

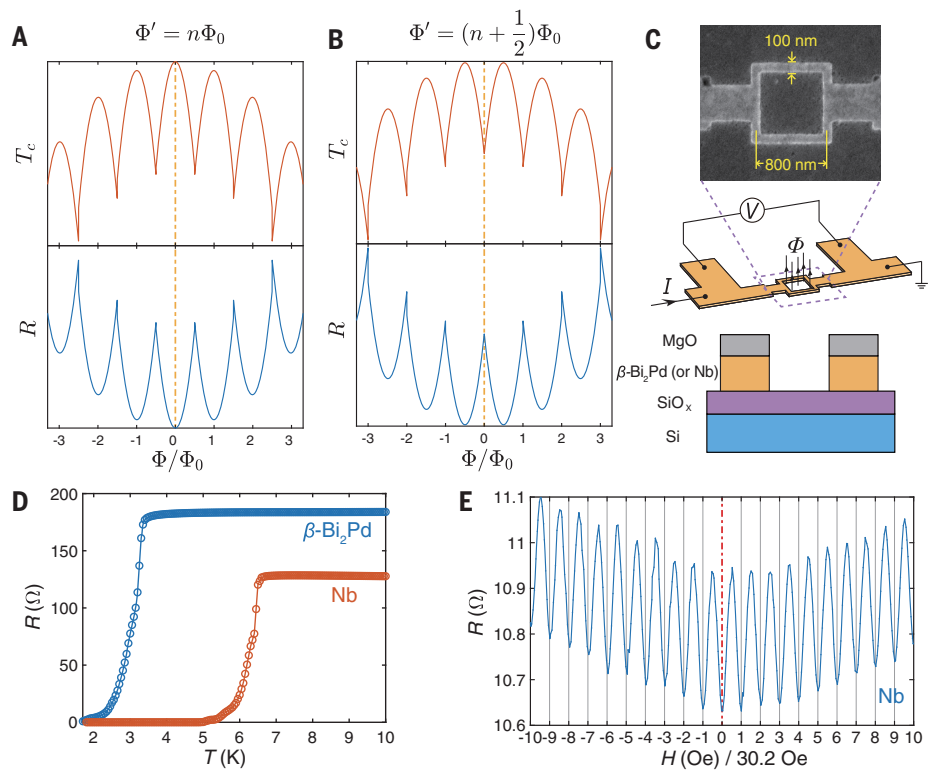
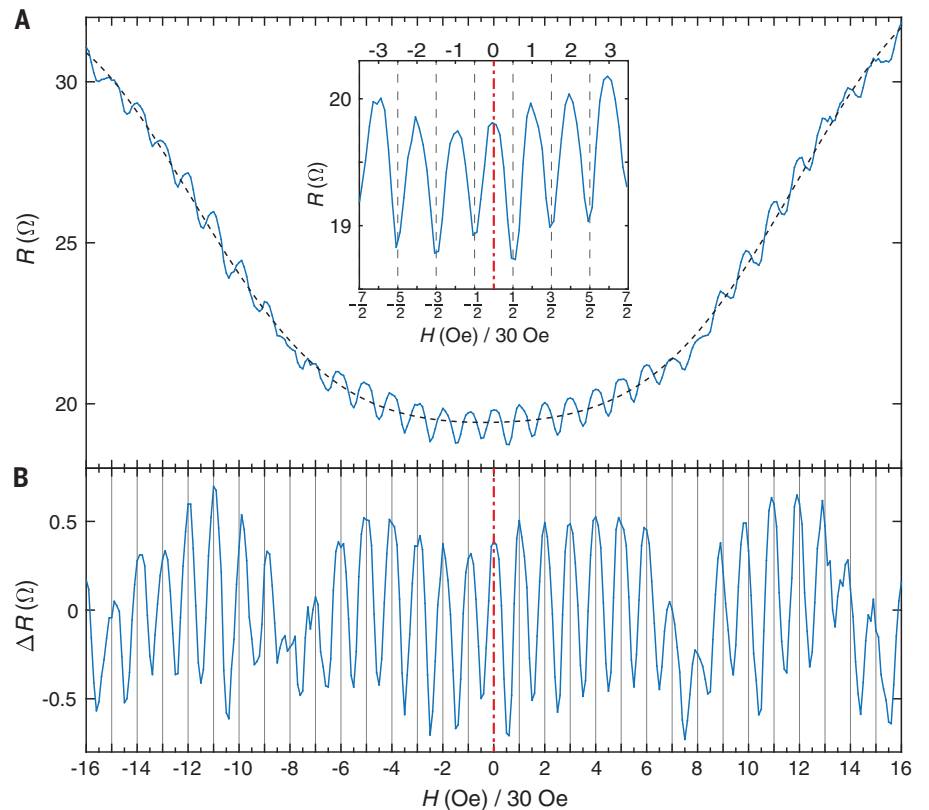


Fig. 2. Little-Parks effect of the β -Bi₂Pd ring device. (A) Oscillations of the β -Bi₂Pd ring device resistance as a function of the perpendicularly applied magnetic field. The sample was held at a constant temperature of 2.5 K. The x-axis is displayed in the units of the oscillation period 30 Oe, in agreement with the expected magnetic flux quantum for the device geometry (800 nm by 800 nm in area). The black dashed line denotes the aperiodic background. The inset is a magnified view of the low field region of (A). The vertical black dashed lines denote the applied magnetic flux of $\Phi = (n + 1/2)\Phi_0$, which corresponds to the oscillation minima. The red dot-dashed line denotes the zero external field. (B) Little-Parks oscillation in which the background has been subtracted from the raw data as presented in (A). The gray vertical lines denote the applied magnetic flux of $\Phi = n\Phi_0$, which corresponds to the oscillation maxima.



resistance (5). Figure 1E presents a typical result of the Little-Parks experiment obtained from the Nb ring device. The observed oscillation period is 30.2 Oe, in good agreement with 32.3 Oe as expected for Φ_0 of the 800 nm by

800 nm ring area. The resistance minima, which correspond to the T_c maxima, occur when $\Phi = n\Phi_0$, as routinely observed for s-wave SCs (6) and high- T_c cuprate d-wave SCs (7, 37), both singlet SCs. Up to ~70 oscillations have been

observed on a roughly parabolic-shaped background. The background is commonly observed in Little-Parks experiments, believed to originate from the misalignment of the magnetic field and the finite line width of the ring

(6, 38, 39). As a precaution against trapping vortices, the measurements were always performed after zero-field cooling from 10 K.

The Little-Parks oscillations of the β -Bi₂Pd ring device are shown in Fig. 2A. The same oscillation period of ~ 30 Oe is observed. A smooth background can be subtracted from the raw data (36), and the resulting ΔR versus H oscillations are presented in Fig. 2B. The magnitude of the resistance oscillations translates to ~ 0.015 K variations of T_c , consistent with theoretical expectations for the Little-Parks effect (40). In stark contrast to the case of Nb, however, $\Phi = n\Phi_0$ now corresponds to the resistance maxima (or the T_c minima); the resistance minima and the T_c maxima are observed when the applied magnetic flux is $(n + 1/2)\Phi_0$. The $\frac{1}{2}\Phi_0$ shift of the free-energy minima indicates that the fluxoid quantization of $(n + 1/2)\Phi_0$ is energetically preferred. When the external magnetic field is zero, the superconducting ring circulates a finite $\vec{j}_s$ to sustain one HQF, which accounts for the lowest T_c and the highest free energy. The system has a lower energy when the external field supplies $\pm \frac{1}{2}\Phi_0$ and $\vec{j}_s$ can become zero. The shape of the magnetoresistance oscillation trace is symmetric with respect to the zero magnetic field, which shows that the effect is not caused by defect-trapped vortices. We also verify that the result is robust against the different field-sweeping directions and current densities (36). The HQF is repeatedly observed in numerous β -Bi₂Pd rings with various geometrical device shape factors (36).

In Fig. 3, we demonstrate the temperature dependence of the Little-Parks effect in β -Bi₂Pd. The π phase shift persists from the emergence of the Little-Parks effect at 2 K, through the highest temperature of 2.6 K, before the oscillation disappears presumably owing to the loss of coherence over the length scale of the ring device. This persistence suggests the predominance of the characteristic pairing symmetry that gives rise to the HQF (41).

Below, we discuss the implications of our experimental observations. The GLB HQF is an experimental signature for unconventional pairing mechanisms, not restricted to the p-wave pairing; indeed, HQF was first observed in d-wave SC tricrystals (12). The orientations of the crystal boundaries had to be carefully designed or HQF would not exist (12, 13). HQF has not been observed in polycrystalline specimens of high- T_c SCs, although the Little-Parks experiment has been conducted on microstructured mesh of polycrystalline YBa₂Cu₃O₇ thin films (7). By contrast, in our experiment, HQFs were observed in most of the β -Bi₂Pd ring devices (36). Considering the previous literature reporting the link between β -Bi₂Pd and the spin-triplet pairing (29–31), a contributing factor to the robustness of the

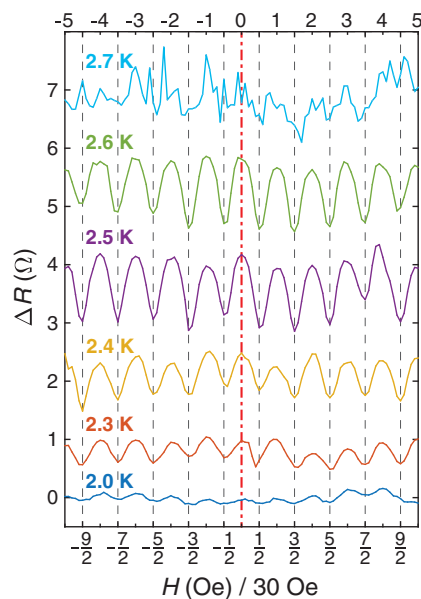


Fig. 3. Temperature evolution of the Little-Parks effect of the β -Bi₂Pd ring device. Oscillations of the resistance as a function of applied magnetic field with the background subtracted for six different values of temperature are shown. The vertical black dashed lines denote the applied magnetic flux of $\Phi = (n + 1/2)\Phi_0$. Results are for the same device presented in Fig. 2 (800 nm by 800 nm in area).

HQF state might be the characteristic anisotropy of the p-wave pairing. The order parameter reverses sign when rotating 180°, whereas for the d-wave, the sign reversal occurs upon rotating 90°. This protects the HQF from moderate disorders on the crystal grain boundaries compared with that gauged for d-wave SCs (12, 13). Nevertheless, the observation of HQF is unequivocal evidence for unconventional superconductivity and is consistent with p-wave pairing in β -Bi₂Pd.

The convenient realization of HQFs in polycrystalline β -Bi₂Pd may find immediate applications in superconducting quantum bits (qubits). In flux qubits, two quantum states with opposite directions of circulating supercurrent serve as the basis states (42). Implementation of flux qubits has been demonstrated using conventional s-wave SCs (43). The operation of such qubits requires an external magnetic field to supply precisely $\Phi = 1/2\Phi_0$, to create the superposition of the two quantum basis states. This requirement is specific to a particular qubit on the basis of its dimensions, hindering device integration. By contrast, a superconducting ring of β -Bi₂Pd hosts HQF as its ground state; in the absence of any external field, it is spontaneously a superposition of two quantum basis states with left-handed and right-handed circulating supercurrent. Such a field-free qubit may enable

practical application of flux qubits for quantum computing.

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the devices. Y.L. and X.X. conducted the electrical transport measurements. M.-H.L. and M.-W.C. conducted the cross-section TEM measurements. Y.L., X.X., and C.L.C. jointly discussed the results and wrote the manuscript with contributions from all authors.

Competing interests: The authors declare no competing interests. Y.L., X.X., and C.L.C. are authors of a patent application filed through Johns Hopkins University that relates to this work (application no.

62/847,028 filed 13 May 2019). **Data and materials availability:** Data reported in this paper are archived online at Harvard Dataverse (44).

SUPPLEMENTARY MATERIALS

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Materials and Methods

Supplementary Text
Figs. S1 to S9
References (45–47)

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GAS SEPARATIONS

Synergistic sorbent separation for one-step ethylene purification from a four-component mixture

Kai-Jie Chen^{1*†}, David G. Madden^{2*†}, Soumya Mukherjee², Tony Pham^{3§}, Katherine A. Forrest³, Amrit Kumar², Brian Space³, Jie Kong¹, Qiu-Yu Zhang¹, Michael J. Zaworotko^{2†}

Purification of ethylene (C_2H_4), the largest-volume product of the chemical industry, currently involves energy-intensive processes such as chemisorption (CO_2 removal), catalytic hydrogenation (C_2H_2 conversion), and cryogenic distillation (C_2H_6 separation). Although advanced physisorbent or membrane separation could lower the energy input, one-step removal of multiple impurities, especially trace impurities, has not been feasible. We introduce a synergistic sorbent separation method for the one-step production of polymer-grade C_2H_4 from ternary ($C_2H_2/C_2H_6/C_2H_4$) or quaternary ($CO_2/C_2H_2/C_2H_6/C_2H_4$) gas mixtures with a series of physisorbents in a packed-bed geometry. We synthesized ultrasensitive microporous metal-organic materials that were readily regenerated, including one that was selective for C_2H_6 over CO_2 , C_2H_2 , and C_2H_4 .

Purification of commodities currently consumes 15% of global energy, and commodity demand has been projected to triple by 2050. The production of ethylene (C_2H_4) and propylene (C_3H_6) uses 0.3% of global energy production (1) as polymer-grade (>99.9% purity) C_2H_4 is produced by energy-intensive separation of downstream C_2 hydrocarbon gas mixtures during the steam cracking process. Acetylene (C_2H_2) is removed through catalytic hydrogenation (with a noble-metal catalyst at high temperature and pressure) or solvent extraction (requiring a large volume of solvent and a large plant installation). Removal of C_2H_6 occurs through cryogenic distillation (2).

The high energy footprint associated with C_2H_4 production has spurred research into the development of more energy-efficient approaches to purification of these C_2 gases. To afford polymer-grade C_2H_4 in a single step, simultaneous removal of C_2H_2 and C_2H_6 from C_2H_4 would be necessary. Chemical transformation of C_2H_2 and C_2H_6 to C_2H_4 , chemisorption, extraction, and membrane-based technologies could in principle address the need, but each approach has drawbacks. The simultaneous separation of C_2H_2 , C_2H_6 , and other trace impurities from C_2H_4 with a physisorbent could enhance the energy efficiency of C_2H_4 production but is too challenging for classical physisorbents. The fundamental limitation

lies in the respective quadrupole moments and kinetic diameters of the C_2 gases; C_2H_4 (1.5×10^{-26} esu cm^2 and 4.1 Å) lies just between C_2H_2 (7.2×10^{-26} esu cm^2 and 3.3 Å) and C_2H_6 (0.65×10^{-26} esu cm^2 and 4.4 Å), which precludes most physisorbents from being highly selective (3).

The task is compounded when CO_2 (4.3×10^{-26} esu cm^2 and 3.3 Å) is also an impurity in a C_2 gas mixture, because a physisorbent would then require strong affinity toward C_2H_2 , C_2H_6 , and CO_2 versus C_2H_4 . Metal-organic materials, also known as metal-organic frameworks (MOFs) or porous coordination polymers, have gained attention for gas separations because of their tunability over pore size and pore chemistry (4–9). Several studies have addressed the separation of C_2H_2 , C_2H_4 , C_2H_6 , and CO_2 by physisorbents (e.g., zeolites, activated car-

bon, porous organic frameworks, and MOFs) (10–17), but selectivity that realizes polymer-grade C_2H_4 production from a quaternary gas mixture (C_2H_2 – C_2H_4 – C_2H_6 – CO_2) is still an important goal. Indeed, even for the ternary C_2H_2 – C_2H_4 – C_2H_6 mixture, only a recent report by Lu and co-workers found discrimination toward C_2H_4 over C_2H_2 and C_2H_6 in the MOF material TJT-100 (18). The task is further compounded for wet gas streams because water vapor can interfere with physisorbent performance through co-adsorption or hydrolytic degradation (19). This would necessitate pretreatment of a gas mixture with a desiccant material.

We addressed this separation challenge by developing synergistic sorbent separation technology (SSST), which uses the favorable sorption properties of task-specific ultramicroporous physisorbents, each with ultrahigh selectivity for one of the impurities, to enable one-step production of C_2H_4 from C_2 gas mixtures (Fig. 1A). Such an approach was used to address the desulfurization of hydrocarbons, but sulfur impurities with strong polarity are much easier to isolate from hydrocarbons (20). With respect to C_2H_2 and C_2H_6 removal from C_2H_4 streams, TIFSIX-2-Cu-i (TIFSIX = TiF_6^{2-} , 2 = 4,4'-dipyridylacetylene, i = interpenetrated) offers ultrasensitive C_2H_2 capture (21, 22) and, as shown below, Zn-atz-ipa (atz = 3-amino-1,2,4-triazolate; ipa = isophthalate) (23), exhibits strong affinity for C_2H_6 over C_2H_4 and the other impurities. In principle, a combination of these two physisorbents could synergistically capture C_2H_2 and C_2H_6 in a tandem-packed sorbent bed to produce polymer-grade (>99.9%) C_2H_4 via physisorption. Further,

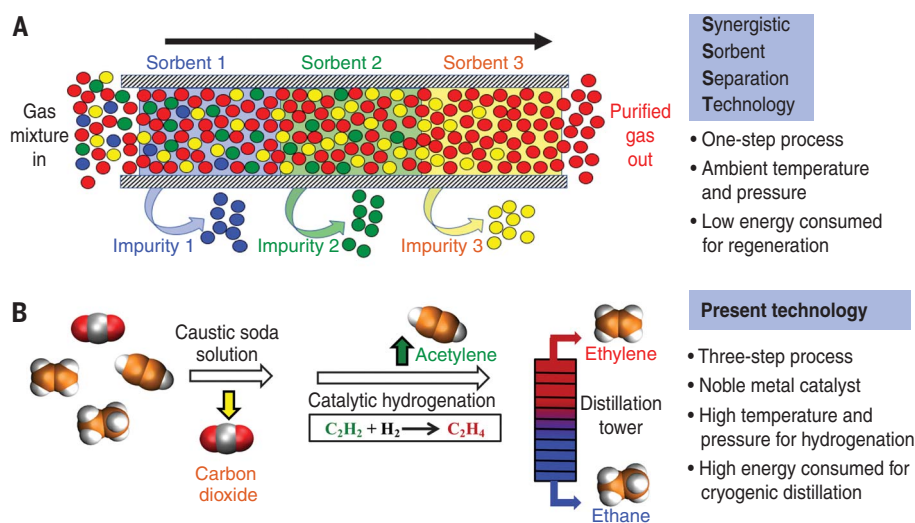


Fig. 1. Synergistic sorbent separation technology (SSST) versus present approaches to purify C_2H_4 . (A) SSST involves an adsorption bed with three task-specific physisorbents to purify the commodity (red) with specific binding sites for each trace impurity (blue, green, yellow). (B) The present process for producing polymer grade C_2H_4 involves three energy-intensive steps.

¹Department of Applied Chemistry, School of Natural and Applied Sciences, Northwestern Polytechnical University, Xi'an, Shaanxi 710072, P.R. China. ²Bernal Institute and Department of Chemical Sciences, University of Limerick, Limerick V94 T9PX, Republic of Ireland. ³Department of Chemistry, University of South Florida, Tampa, FL 33620, USA.

*These authors contributed equally to this work.

†Corresponding author. Email: ckjison@nwpu.edu.cn (K.-J.C.); michael.zaworotko@ul.ie (M.J.Z.) ‡Present address: Department of Chemical Engineering and Biotechnology, University of Cambridge, Cambridge CB3 0AS, UK. §Present address: Department of Chemistry, Biochemistry, and Physics, University of Tampa, Tampa, FL 33606, USA.

the addition of SIFSIX-3-Ni (SIFSIX = SiF_6^{2-} , 3 = pyrazine), an ultrasensitive CO_2 sorbent (19, 24), to the sorbent bed should enable removal of trace levels of CO_2 from the corresponding four-component gas mixture, thereby offering a one-step process that offers advantages over present approaches (Fig. 1). The selected physisorbents are stable to humidity, but their sorption performance is reduced in the presence of water vapor (19, 25).

TIFSIX-2-Cu-i and SIFSIX-3-Ni belong to a family of hybrid ultramicroporous materials of general formula $\text{M}'\text{SIFSIX-L-M}$ (M = divalent transition metal center; L = dipyrindyl organic linker; $\text{M}' = \text{Si, Ti, Ge, Zr, Sn}$). In this family, square lattice networks are formed by transition metal nodes and bipyridyl-type linkers, which are pillared by inorganic linkers to generate primitive cubic topology coordination networks with tunable pore size and chemistry (21, 24).

TIFSIX-2-Cu-i and SIFSIX-3-Ni have previously been shown to exhibit benchmark performance for trace C_2H_2 (21) and trace CO_2 sorption, respectively (19, 26), but their pore size and chemistry renders them ill-suited for C_2H_6 -selective sorption. Indeed, we are unaware of any existing sorbents that are even mildly selective toward C_2H_6 over CO_2 , C_2H_2 , and C_2H_4 (table S1).

The ultramicroporous MOF Zn-atz-ipa was selected in this context given its excellent water stability and unusual pore chemistry (23); as detailed below, this pore chemistry makes it suitable for the intended purpose. To evaluate the selected sorbents for C_2H_4 purification, we first determined their pure gas adsorption properties. Each sorbent was synthesized according to previous reports (19, 22, 23). To verify purity, we collected powder x-ray diffraction (XRD) patterns and sorption data at cryogenic

temperatures on as-synthesized materials after activation (figs. S1 and S2). Single-gas isotherms at 273 and 298 K were collected to 1 bar for TIFSIX-2-Cu-i, SIFSIX-3-Ni, and Zn-atz-ipa (figs. S3 to S5). As shown in Fig. 2B, at 298 K and 1 bar, TIFSIX-2-Cu-i exhibited less uptake for C_2H_6 (2.1 mmol/g) than for C_2H_4 (2.6 mmol/g), CO_2 (4.3 mmol/g), and C_2H_2 (4.1 mmol/g). C_2H_2 exhibited the highest uptake from 0 to 0.8 bar for TIFSIX-2-Cu-i. In the case of SIFSIX-3-Ni, CO_2 exhibited the highest uptake at 298 K below 0.2 bar (Fig. 2C). For Zn-atz-ipa, all four gases showed similar uptake (1.8 to 2.0 mmol/g) at 1 bar and 298 K (Fig. 2A). However, from 0 to 0.4 bar, we measured higher uptake for C_2H_6 over CO_2 , C_2H_2 , and C_2H_4 .

To quantify the strength of sorbent-sorbate interactions, we fit 273 and 298 K sorption data by the virial equation (figs. S6 to S11) and

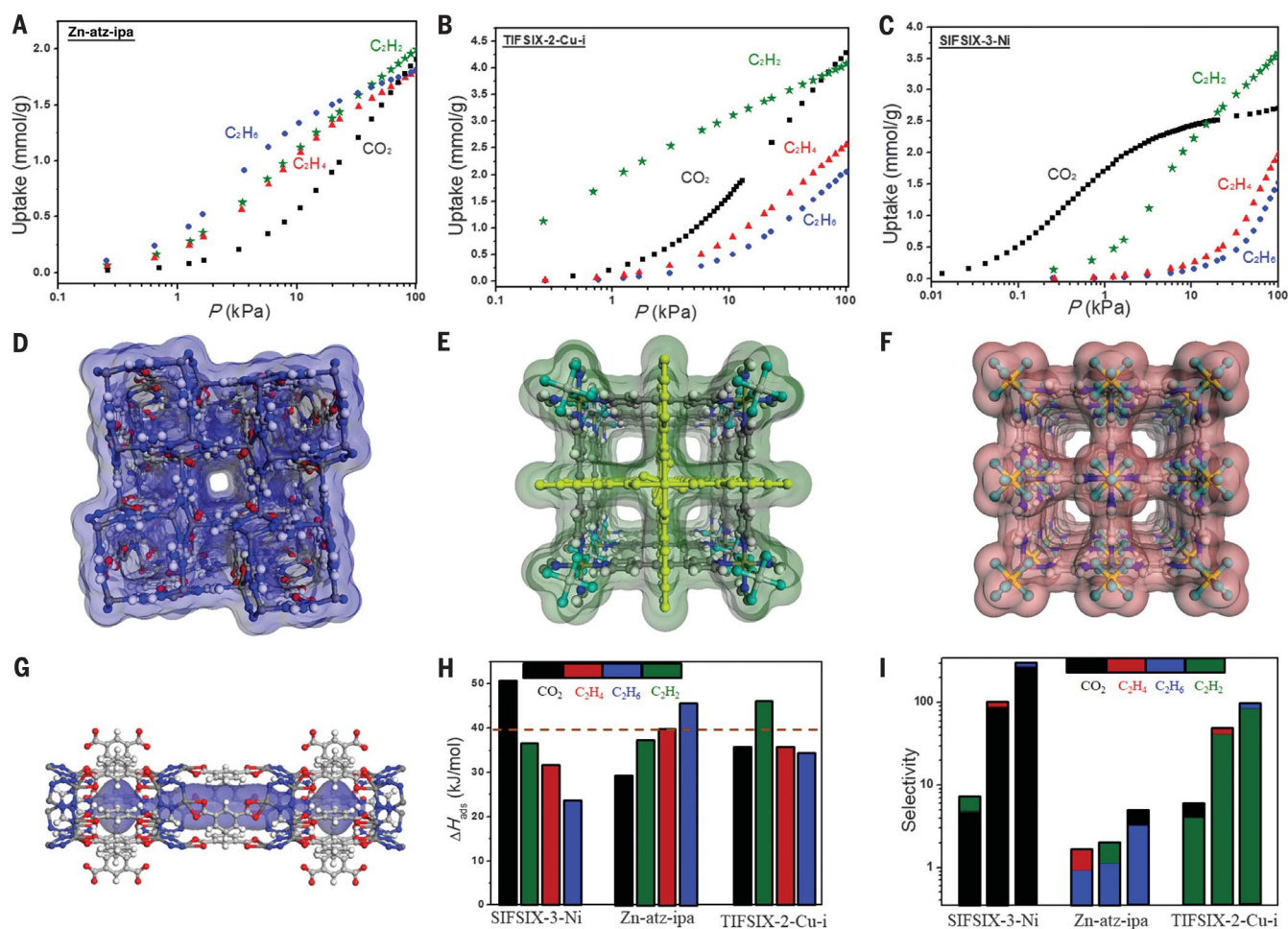


Fig. 2. Structures of physisorbents used herein and their single component gas sorption properties. (A to C) Adsorption of CO_2 (black squares), C_2H_2 (green stars), C_2H_4 (red triangles), and C_2H_6 (blue circles) at 298 K for Zn-atz-ipa (A), TIFSIX-2-Cu-i (B), and SIFSIX-3-Ni (C). (D to F) Structures of Zn-atz-ipa (D), TIFSIX-2-Cu-i (E), and SIFSIX-3-Ni (F). (G) The Connolly surface of Zn-atz-ipa with probe radius of 2.0 Å. (H) Isothermic heat of

CO_2 (black), C_2H_2 (green), C_2H_4 (red), and C_2H_6 (blue) in SIFSIX-3-Ni, Zn-atz-ipa, and TIFSIX-2-Cu-i. The "strong binding" threshold of 40 kJ/mol is highlighted with a dashed line. (I) Selectivity for adsorbates in SIFSIX-3-Ni (CO_2 over $\text{C}_2\text{H}_2/\text{C}_2\text{H}_4/\text{C}_2\text{H}_6$), Zn-atz-ipa (C_2H_6 over $\text{C}_2\text{H}_4/\text{C}_2\text{H}_2/\text{CO}_2$), and TIFSIX-2-Cu-i (C_2H_2 over $\text{CO}_2/\text{C}_2\text{H}_4/\text{C}_2\text{H}_6$) at 298 K and 1 bar for 1:1 gas mixtures from ideal adsorbed solution theory.

calculated the isosteric heat of adsorption (Q_{st}) according to the Clausius-Clapeyron equation. Q_{st} values at low loading of the four gases in TIFSIX-2-Cu-i, SIFSIX-3-Ni, and Zn-atz-ipa are compared in Fig. 2H. Full Q_{st} curves for the four gases in the three ultramicroporous sorbents are given in figs. S12 to S14 and summarized in table S2. Each sorbent exhibited strong selectivity for one gas over the other three according to Q_{st} : CO_2 @SIFSIX-3-Ni (50.9 kJ/mol), C_2H_6 @Zn-atz-ipa (45.8 kJ/mol), and C_2H_2 @TIFSIX-2-Cu-i (46.3 kJ/mol) (Fig. 2H, dashed line). These results indicate that, at least in principle, CO_2 , C_2H_2 , and C_2H_6 in a four-component gas mixture including C_2H_4 should be selectively captured by SIFSIX-3-Ni, TIFSIX-2-Cu-i, and Zn-atz-ipa, respectively. The pure gas sorption performance of the three selected sorbents therefore met the needed criteria for a one-step SSST process.

The gas adsorption selectivity at 298 K and 1 bar was calculated for pairs of adsorbates in 1:1 gas mixtures using ideal adsorbed solution theory (27, 28). Detailed fitting parameters are provided in figs. S15 to S20 and tables S3 to S5. As shown in Fig. 2I, TIFSIX-2-Cu-i and SIFSIX-3-Ni exhibited high adsorption selectivity for C_2H_2 and CO_2 , respectively, over the other three gases. Indeed, new benchmark selectivity values were found under these or similar conditions (13, 29): $\text{C}_2\text{H}_2/\text{C}_2\text{H}_4$ (49) and $\text{C}_2\text{H}_2/\text{C}_2\text{H}_6$ (98) in

TIFSIX-2-Cu-i; $\text{CO}_2/\text{C}_2\text{H}_4$ (103) and $\text{CO}_2/\text{C}_2\text{H}_6$ (308) in SIFSIX-3-Ni. High selectivity was also calculated for $\text{CO}_2/\text{C}_2\text{H}_2$ (6.1 for $\text{C}_2\text{H}_2/\text{CO}_2$ by TIFSIX-2-Cu-i, 6.9 for $\text{CO}_2/\text{C}_2\text{H}_2$ by SIFSIX-3-Ni) (22). Zn-atz-ipa exhibited selective C_2H_6 adsorption with a selectivity of 1.7 for $\text{C}_2\text{H}_6/\text{C}_2\text{H}_2$, 2 for $\text{C}_2\text{H}_6/\text{C}_2\text{H}_4$, and 5 for $\text{C}_2\text{H}_6/\text{CO}_2$. Thus, although Zn-atz-ipa is a physisorbent, it exhibited selective adsorption of C_2H_6 over C_2H_4 , C_2H_2 , and CO_2 ; we consider this result unexpected because C_2H_6 tends to be weakly adsorbed as a consequence of its low quadrupole moment.

To better understand the interactions of the four gases with the three sorbents, we conducted grand canonical Monte Carlo (GCMC) simulations. Final optimized results for SIFSIX-3-Ni and TIFSIX-2-Cu-i for C_2H_2 and CO_2 were consistent with earlier reports (21, 30). Binding-site information for all gases is given in Fig. 3 and figs. S21 to S24. In SIFSIX-3-Ni, CO_2 binding is driven by interactions with four electro-negative F atoms from four independent SiF_6^{2-} anions. C_2H_2 was trapped through multiple $\text{C-H}\cdots\text{F}$ interactions with $\text{H}\cdots\text{F}$ distances of 3.3 to 4.5 Å between C_2H_2 and eight SiF_6^{2-} anions. In contrast, C_2H_4 and C_2H_6 exhibited simultaneous interactions with two and six SiF_6^{2-} anions, respectively. Although there are fewer contacts with anions, shorter distances of 2.51 and 2.62 Å for C_2H_4 suggest favorable C_2H_4 binding over C_2H_6 (2.59 to 2.76 Å).

The overall trend of adsorption energy from calculations, $\text{CO}_2 > \text{C}_2\text{H}_4 > \text{C}_2\text{H}_2 > \text{C}_2\text{H}_6$, is fully consistent with experimental data. In TIFSIX-2-Cu-i, C_2H_2 , C_2H_4 , and C_2H_6 are localized so that every molecule could interact with two TiF_6^{2-} anions through $\text{C-H}\cdots\text{F}$ interactions. However, C_2H_2 had shorter contacts (2.46 and 2.50 Å) relative to C_2H_4 (2.45 and 2.52 Å) and C_2H_6 (2.62 and 2.90 Å). Moreover, the more acidic C_2H_2 molecule ($\text{p}K_a = 26$, versus C_2H_4 , $\text{p}K_a = 45$, and C_2H_6 , $\text{p}K_a = 62$) would be expected to form stronger hydrogen bonds. For TIFSIX-2-Cu-i, CO_2 molecules interact with two F atoms from one TiF_6^{2-} anion, with short interaction distances between the C atom of CO_2 and the F atoms of the TiF_6^{2-} anion (2.65 and 3.48 Å). The calculated hierarchy in TIFSIX-2-Cu-i was $\text{C}_2\text{H}_2 > \text{CO}_2 > \text{C}_2\text{H}_4 > \text{C}_2\text{H}_6$.

In Zn-atz-ipa, all six H atoms of one C_2H_6 molecule interacted with the pore surface. This tight-fitting binding site helps to explain the high adsorption energy of 42.2 kJ/mol from GCMC calculations, a value near the experimental value of 45.8 kJ/mol. In contrast, smaller molecules such as C_2H_4 , C_2H_2 , and CO_2 only interacted through two or three close contacts, and, although amino groups are considered to improve CO_2 binding, the amino group of the atz ligand was not exposed on the pore surface. Thus, CO_2 binding was weak and the strength of interactions in Zn-atz-ipa

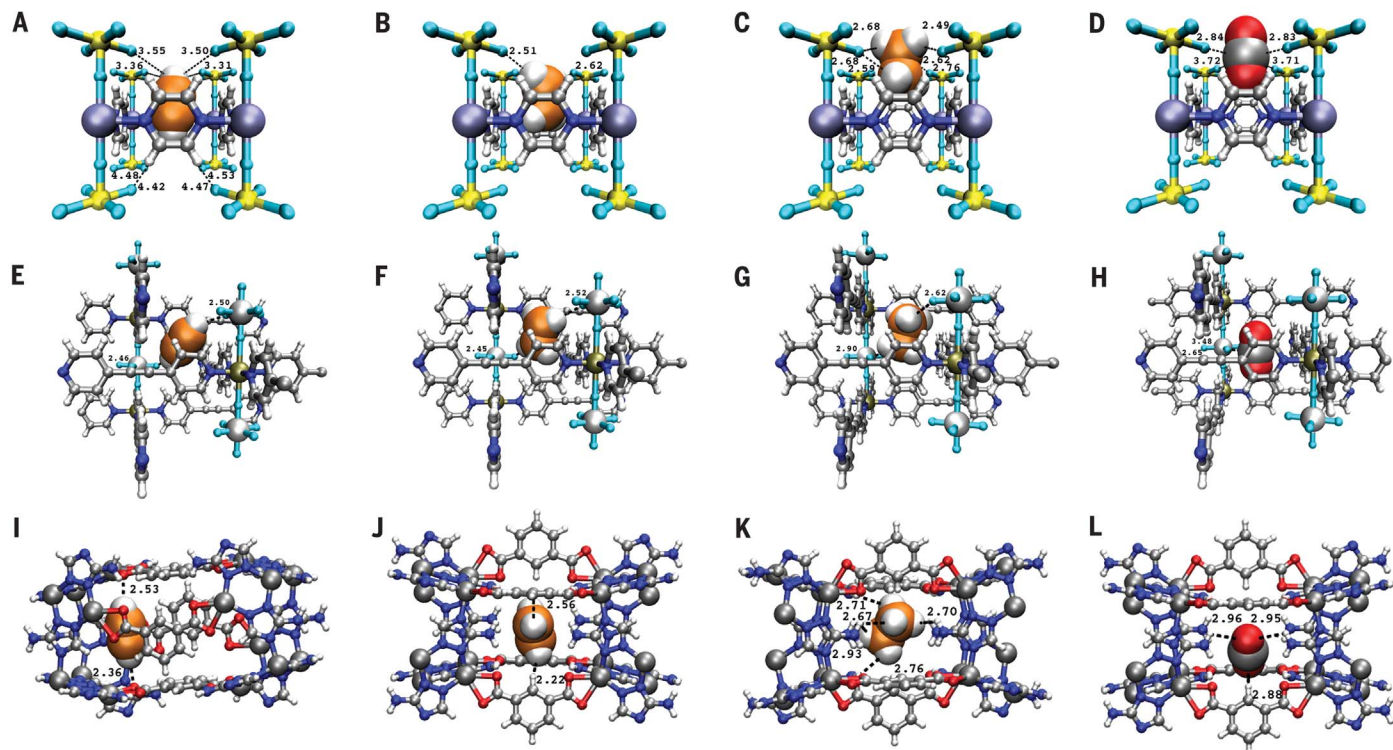


Fig. 3. Molecular simulation and periodic density functional theory calculations. (A to L) C_2H_2 [(A), (E), and (I)], C_2H_4 [(B), (F), and (J)], C_2H_6 [(C), (G), and (K)], and CO_2 [(D), (H), and (L)] binding sites in SIFSIX-3-Ni (top), TIFSIX-2-Cu-i (middle), and Zn-atz-ipa (bottom). Closest contacts between framework atoms and gas molecules are defined by the distance (in angstroms) between the H atom of hydrocarbons and the closest framework atoms. Adsorbed C_2 and CO_2 molecules are presented in space-filling display mode (C, gray; H, white; O, red; N, blue; F, cyan; Si, yellow; Ni, lavender; Ti, silver; Cu, gold; Zn, dark gray).

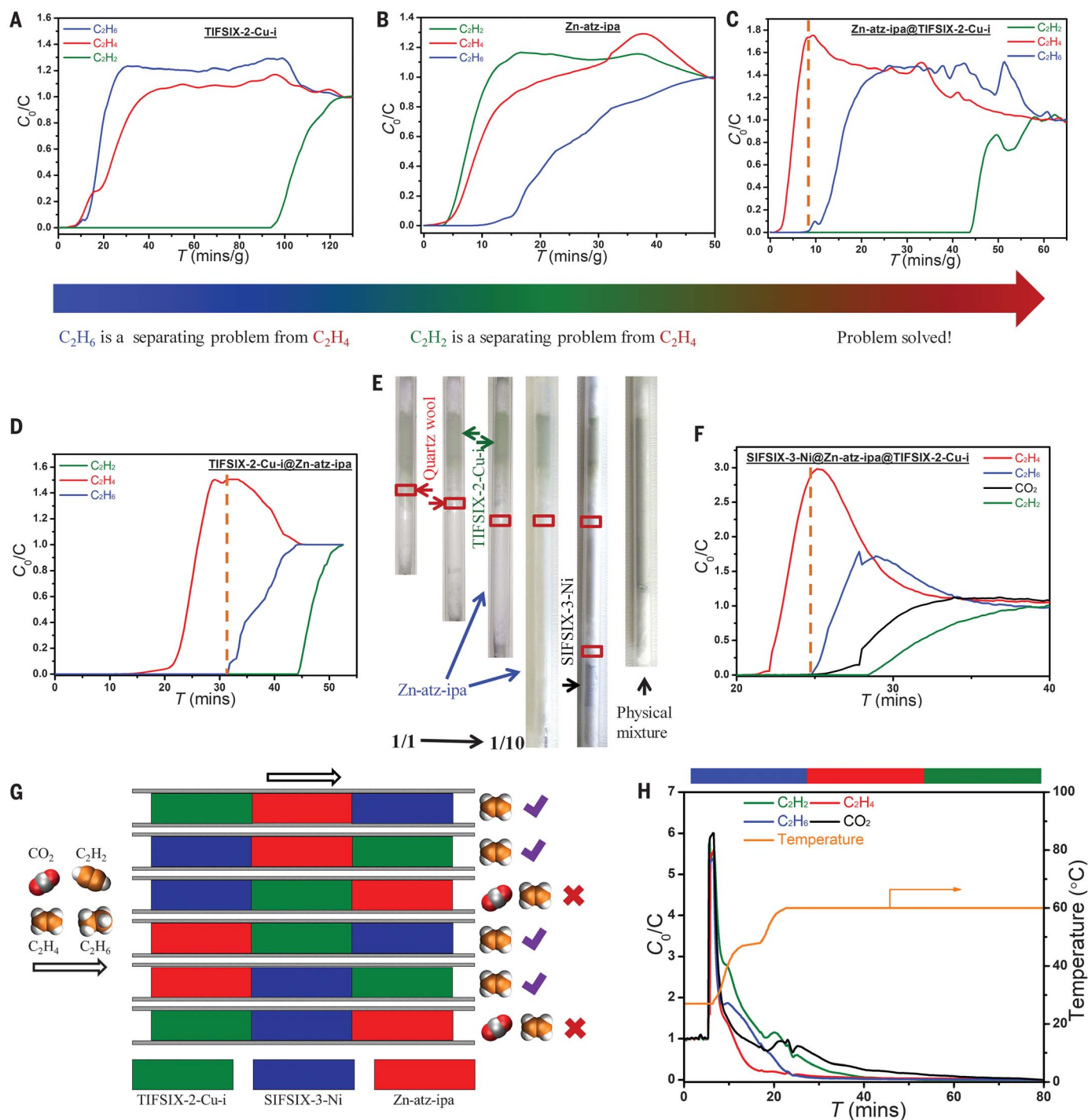


Fig. 4. Experimental column breakthrough results. (A and B) Experimental column breakthrough curves for $C_2H_2/C_2H_4/C_2H_6$ separation (1:1:1 mixture) on TIFSIX-2-Cu-i and Zn-atz-ipa at 298 K and 1 bar. Breakthrough experiments were conducted in a column (inside diameter, 8 mm) at a flow rate of 2.1 ml/min. (C) Experimental column breakthrough curves for $C_2H_2/C_2H_4/C_2H_6$ separation (1:1:1 mixture) on a tandem-packed column of TIFSIX-2-Cu-i (~250 mg) and Zn-atz-ipa (~600 mg) at 298 K and 1 bar. The x axis is displayed as minutes per gram of TIFSIX-2-Cu-i + Zn-atz-ipa. The orange dashed line highlights the cutoff time for C_2H_4 with purity >99.9% in this and other plots. (D) Experimental column breakthrough curves for $C_2H_2/C_2H_4/C_2H_6$ separation (1:49.5:49.5 mixture) on a

tandem-packed column of TIFSIX-2-Cu-i (~120 mg) and Zn-atz-ipa (~1200 mg). (E) SSST sorption beds. From left to right: 1:1 to 1:10 TIFSIX-2-Cu-i + Zn-atz-ipa; 1:1.25:10 TIFSIX-2-Cu-i + Zn-atz-ipa + SIFSIX-3-Ni; physical mixture of TIFSIX-2-Cu-i + Zn-atz-ipa after breakthrough experiments. (F) Experimental column breakthrough curves for $CO_2/C_2H_2/C_2H_4/C_2H_6$ separation (1:1:1:1 mixture) on a tandem-packed column of TIFSIX-2-Cu-i (~120 mg), SIFSIX-3-Ni (~150 mg), and Zn-atz-ipa (~1200 mg) at 298 K and 1 bar (packing order: SIFSIX-3-Ni@Zn-atz-ipa@TIFSIX-2-Cu-i). (G) The effect of packing order of the SSST sorbents on ethylene purity. (H) Temperature-programmed desorption curves recorded on the column in (F) at 60°C under He flow of 20 cm³/min.

followed the sequence $C_2H_6 > C_2H_4 > C_2H_2 > CO_2$. (See supplementary materials for more details on these simulations.)

We conducted dynamic breakthrough experiments at 298 K on a custom-built apparatus (fig. S25) using an equimolar three-component gas mixture of $C_2H_2/C_2H_4/C_2H_6$ at a total pressure of 1 bar. All sorbents were preactivated by heating under high vacuum before preparing the breakthrough column. We first conducted control experiments using sorbent beds of TIFSIX-2-Cu-i or Zn-atz-ipa. C_2H_2 was selectively captured, but C_2H_4 and C_2H_6 were not separated by TIFSIX-2-Cu-i (Fig. 4A). For Zn-atz-ipa (Fig. 4B), C_2H_6 was selectively adsorbed for ~10 min, but C_2H_2 and C_2H_4 were not separated. However, SSST with a two-component (tandem) bed containing TIFSIX-2-Cu-i and Zn-atz-ipa cleanly removed C_2H_2 and C_2H_6 with C_2H_4 at >99.9% purity in the effluent stream (Fig. 4C). By increasing the mass ratio of Zn-atz-ipa over TIFSIX-2-Cu-i from 1:1 to 10:1, breakthrough times of C_2H_2 and C_2H_6 were optimized for the production of pure C_2H_4 using SSST (Fig. 4E and figs. S26 to S29), which suggests that the adsorption capacities of the two adsorbents had been fully used in the case of the 10/1 ratio.

The four-component equimolar mixture of $C_2H_2/C_2H_4/C_2H_6/CO_2$ was studied using SIFSIX-3-Ni in a three-component sorbent bed. A ratio of 1:1.25:10 (TIFSIX-2-Cu-i, 120 mg; Zn-atz-ipa, 1.2 g; SIFSIX-3-Ni, 150 mg) was adopted on the basis of the single-component sorption data. Breakthrough results revealed that CO_2 , C_2H_6 , and C_2H_2 were captured (Fig. 4F), producing polymer-grade C_2H_4 as effluent (working capacity 0.14 mmol/g). The breakthrough sequence follows the order $C_2H_4/C_2H_6/CO_2/C_2H_2$ at 20.3, 24.6, 24.9, and 28.3 min, respectively. Regeneration of the SSST column under He flow (20 ml/min, 1 hour, 60°C) revealed unchanged performance after nine cycles (fig. S30). Tests on the individual sorbents showed facile regeneration with no capacity loss after 10 cycles (figs. S49 to S51). The low energy footprint of the SSST columns was validated by temperature-programmed desorption experiments (Fig. 4H and figs. S52B to S59B).

In industrial C_2 hydrocarbon gas streams, C_2H_2 typically makes up only ~1% of the total flow. To examine the performance of SSST with more industrially relevant and challenging gas mixtures, we also tested $C_2H_2/C_2H_4/C_2H_6$ (1:49.5:49.5) and $C_2H_2/C_2H_4/C_2H_6/CO_2$ (1:33:33:33) gas mixtures. Polymer-grade C_2H_4 with working capacities of 0.32 and 0.10 mmol/g was harvested from 1:49.5:49.5 and 1:33:33:33 gas mixtures, respectively (Fig. 4D and fig. S31). The uptake of C_2H_6 revealed by

its pure gas isotherm with Zn-atz-ipa at 0.495 versus 0.33 bar contributed to the higher working capacity of the 1:49.5:49.5 gas mixture.

The effect of packing order on SSST performance was assessed with six parallel SSST columns and breakthrough experiments with a 1:1:1:1 gas mixture at 298 K and 1 bar. The results (table S6) revealed the importance of packing order (Fig. 4G and figs. S55A to S59A). SIFSIX-3-Ni@Zn-atz-ipa@TIFSIX-2-Cu-i afforded the highest working capacity (0.14 mmol/g), whereas two other combinations, both with Zn-atz-ipa as the final sorbent (SIFSIX-3-Ni@TIFSIX-2-Cu-i@Zn-atz-ipa and TIFSIX-2-Cu-i@SIFSIX-3-Ni@Zn-atz-ipa), failed (Fig. 4H). The effects of different selectivity values, kinetics, and co-adsorption (22) were likely the cause of this observation. Particle size and amount of sorbent had little effect, with smaller particle size and larger sample amounts resulting in slightly improved C_2H_4 purification (figs. S33 to S47). A column with looser packing, however, offered much-reduced performance (fig. S48). The use of a physical mixture also failed. When we used 120 mg of TIFSIX-2-Cu-i and 1200 mg of Zn-atz-ipa on an equimolar gas mixture of $C_2H_2/C_2H_4/C_2H_6$, C_2H_2 was not effectively removed before C_2H_4 breakthrough (fig. S32). Further, C_2H_6 concentration was not reduced to the required specification of <0.1%.

We were able to take advantage of the variations in pore geometry and pore chemistry of three ultramicroporous sorbents to address one-step C_2H_4 purification using SSST. The choice of task-specific ultrasensitive sorbents in tandem-packed sorbent beds of the type used here is unlikely to be limited to the three sorbents or target gas we investigated. Sorbents with higher selectivity, higher uptake capacity, or both could likely be substituted to optimize overall performance. The strong performance of SSST with respect to the purification of C_2 gas mixtures and the availability of an ever-increasing number of ultrasensitive physisorbents suggests that the scope of SSST is likely to be broad enough to address the high energy footprint of other industrial commodity purifications.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S59
Tables S1 to S6
References (31–44)

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Shisa7 is a GABA_A receptor auxiliary subunit controlling benzodiazepine actions

Wenyan Han¹, Jun Li^{1*}, Kenneth A. Pelkey^{2*}, Saurabh Pandey¹, Xiumin Chen³, Ya-Xian Wang⁴, Kunwei Wu¹, Lihao Ge⁵, Tianming Li¹, David Castellano¹, Chengyu Liu⁶, Ling-Gang Wu⁵, Ronald S. Petralia⁴, Joseph W. Lynch³, Chris J. McBain², Wei Lu^{1†}

The function and pharmacology of γ -aminobutyric acid type A receptors (GABA_ARs) are of great physiological and clinical importance and have long been thought to be determined by the channel pore-forming subunits. We discovered that Shisa7, a single-passing transmembrane protein, localizes at GABAergic inhibitory synapses and interacts with GABA_ARs. Shisa7 controls receptor abundance at synapses and speeds up the channel deactivation kinetics. Shisa7 also potentially enhances the action of diazepam, a classic benzodiazepine, on GABA_ARs. Genetic deletion of Shisa7 selectively impairs GABAergic transmission and diminishes the effects of diazepam in mice. Our data indicate that Shisa7 regulates GABA_AR trafficking, function, and pharmacology and reveal a previously unknown molecular interaction that modulates benzodiazepine action in the brain.

Gamma-aminobutyric acid type A receptors (GABA_ARs) mediate fast inhibitory transmission in the mammalian brain and are ligand-gated pentameric anion channels assembled from various combinations of 19 subunits (1, 2). The abundance and kinetics of GABA_ARs at synapses fundamentally control inhibitory synapse strength and neural circuit information processing (3–6). Whether native GABA_ARs contain additional auxiliary subunits that control both trafficking and kinetics of the receptor remains unknown. GABA_ARs are also the primary targets for a number of drugs, notably benzodiazepines (BDZs), barbiturates, anesthetics, and ethanol (7–10).

We performed an immunocytochemical assay to examine the subcellular distributions of Shisa family proteins [Shisa6 to -9; also called cystine-knot AMPA receptor-modulating proteins (CKAMPs)] (fig. S1A) in hippocampal neurons, some of which have been implicated in the regulation of glutamatergic transmission (11, 12), and we unexpectedly identified Shisa7 as a membrane protein localized at GABAergic inhibitory synapses. Whereas overexpressed Shisa6 and Shisa9 were localized at glutamatergic synapses (Fig. 1, A and B, and

fig. S1, B, D, and E), overexpressed Shisa7 largely colocalized with GABA_AR, vesicular GABA transporter (vGAT), or gephyrin puncta (Fig. 1, A and B, and fig. S2, A, B, and E) but not with vesicular glutamate transporter 1 (vGluT1), PSD-95, Homer1, or GluA1 puncta (figs. S1, B and C, and S2, C to F). Recombinant Shisa7 expressed in Shisa7 knockout (KO) neurons colocalized with gephyrin (fig. S2G). Moreover, a monoclonal antibody against Shisa7 confirmed robust colocalization of endogenous Shisa7 with gephyrin (Fig. 1C and fig. S2, H and I). Finally, super-resolution microscopy analysis colocalized Shisa7 with GABA_ARs in the nanometer range (Fig. 1D and figs. S3, A to C, and S4, A to F).

The unexpected localization of Shisa7 at GABAergic synapses prompted further investigation into a potential role in regulating inhibitory transmission. We used two different single-guide RNAs (sgRNA^{#1} and sgRNA^{#2}; see supplementary materials) in this part of our study. sgRNA^{#1}-mediated single-cell genetic deletion of Shisa7 in CA1 pyramidal neurons in acute hippocampal slices produced a strong reduction in frequency, but not amplitude, of miniature inhibitory postsynaptic currents (mIPSCs) (Fig. 1E and fig. S5, A to C). Furthermore, dual whole-cell recordings from neighboring transfected and nontransfected CA1 pyramidal neurons revealed significantly reduced evoked IPSCs in neurons expressing sgRNA^{#1}, but not in neurons expressing sgRNA^{#2} (Fig. 1F and figs. S5, A to C, and S6A). IPSC paired-pulse ratios and excitatory transmission were not altered in neurons expressing sgRNA^{#1} (fig. S6, B to F). The reduction of IPSCs in neurons expressing sgRNA^{#1} could be fully rescued by coexpressing the sgRNA^{#1}-resistant Shisa7 (Shisa7*; fig. S6, G to I). In Shisa7 KO mice (fig. S7, A to D), mIPSC frequency, but not amplitude, was strongly decreased in CA1 pyramidal neu-

rons (fig. S7E). Morphologically, interneurons generally segregate into perisomatic and dendrite-targeting subsets (13). To determine if Shisa7 regulates inhibitory inputs on both of these cellular compartments, we examined GABAergic transmission in paired recordings between synaptically coupled stratum radiatum interneurons and CA1 pyramidal neurons, targeting cholecystokinin-expressing basket and dendrite-targeting cells (CCKBCs and CCKDTs, respectively) (Fig. 1G). Shisa7 KO reduced unitary IPSC (uIPSC) amplitudes at both the CCKBC and CCKDT inputs to CA1 pyramidal neurons (Fig. 1H).

Coimmunoprecipitation (co-IP) experiments in human embryonic kidney (HEK) cells revealed that α 1, α 2, and γ 2 subunits, but not β 2 and β 3 subunits, coimmunoprecipitated with Shisa7 (Fig. 2A and fig. S8, A and B). Furthermore, α 1 and α 2 were detected in Shisa7 immunoprecipitates from detergent-solubilized hippocampal lysates prepared from wild-type (WT) mice but not Shisa7 KO mice (fig. S8C). Similarly, a reverse co-IP confirmed an association between Shisa7 and α 1 or α 2 subunits in hippocampal lysates (Fig. 2B and fig. S8D), consistent with findings of a recent proteomic study (14).

We swapped domains of Shisa9, which normally localizes to glutamatergic synapses (fig. S1D) (12), with those of Shisa7 and discovered that the Shisa7 N terminus was critical for its subcellular localization (fig. S9, A to C). Sequence alignment of Shisa6 to -9 revealed a distinctive 22-amino acid domain in the Shisa7 N terminus (fig. S9D). Deletion of this domain (Δ N154-175-Shisa7) largely abolished the colocalization of Shisa7 with gephyrin (fig. S9E). Finally, both Shisa7 and Shisa7- Δ C, which lacks the C terminus, but not Δ N154-175-Shisa7, coimmunoprecipitated with α 2 (Fig. 2C), suggesting that this domain (the GABA_AR interacting domain, or GRID) is critical for the Shisa7-GABA_AR interaction. Finally, cell adhesion assay results supported a direct interaction between Shisa7 and GABA_ARs (fig. S10).

What are the mechanisms underlying the regulation of GABAergic transmission by Shisa7? In HEK cells, Shisa7 significantly promoted cell surface expression of α 2 β 3 γ 2 receptors but not α 1 β 2 γ 2 receptors (fig. S11, A and B). The GRID was critical for Shisa7 to promote GABA_AR trafficking to the cell surface (fig. S11C). Similarly, Shisa7 significantly increased GABA_AR-mediated whole-cell currents evoked by GABA (10 mM) in cells expressing α 2 β 3 γ 2 without affecting the GABA median effective concentration (fig. S11, D and E). Although β 3 on its own did not associate with Shisa7 (fig. S8A), Shisa7 promoted GABA-evoked whole-cell currents mediated by GABA_ARs containing β 3, no matter which α subunit was present (fig. S11D). Conversely, single-cell genetic deletion or germline KO of Shisa7 significantly

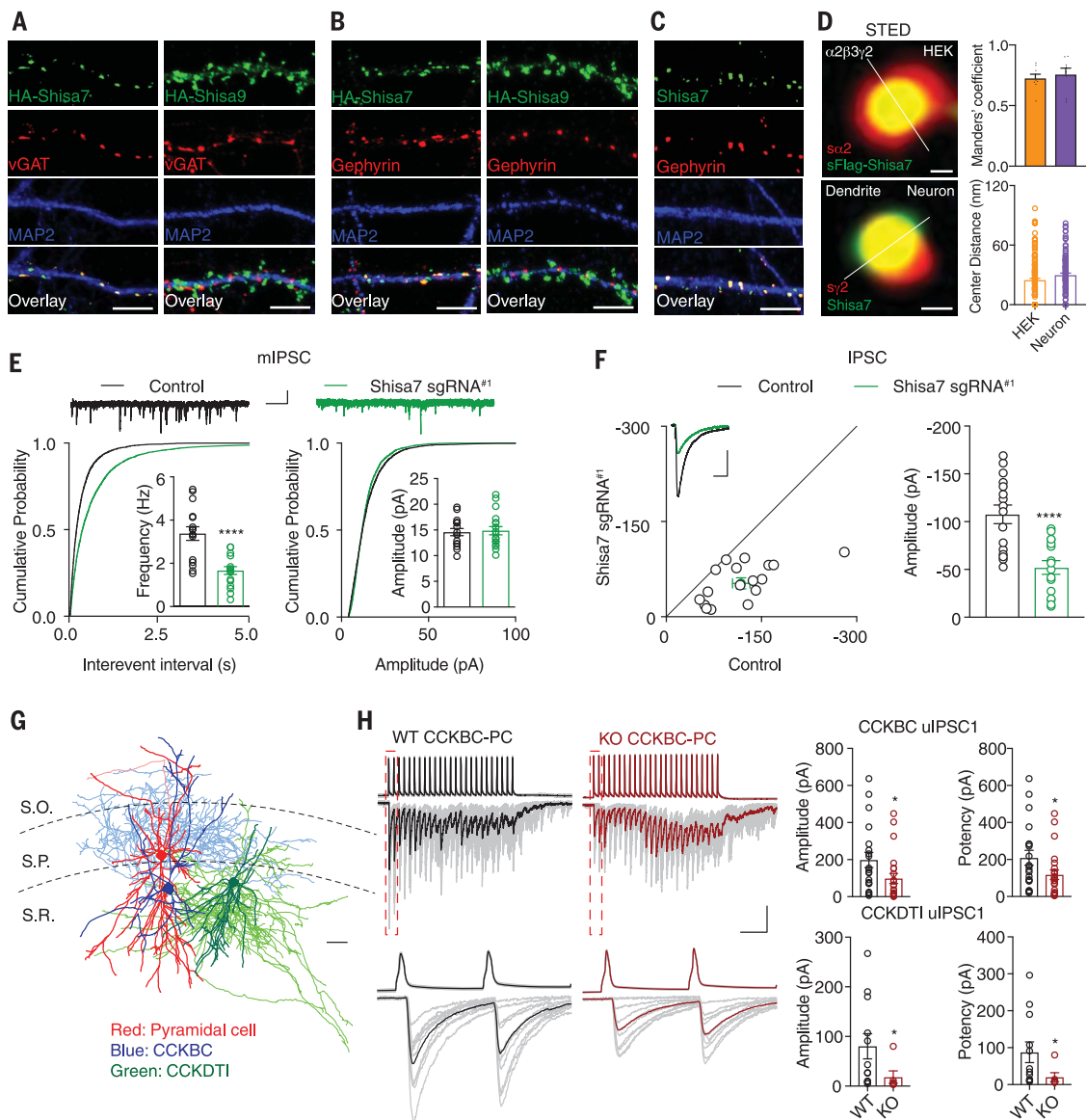
¹Synapse and Neural Circuit Research Unit, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892, USA. ²Cellular and Synaptic Neuroscience Section, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA. ³Queensland Brain Institute, The University of Queensland, Brisbane, QLD 4072, Australia. ⁴Advanced Imaging Core, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, MD 20892, USA. ⁵Synaptic Transmission Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892, USA. ⁶Transgenic Core Facility, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA.

*These authors contributed equally to this work.

†Corresponding author. Email: luw4@mail.nih.gov

Fig. 1. Identification of Shisa7 as a critical molecule for inhibitory transmission. (A and B) HA-Shisa7, but not HA-Shisa9, colocalized with vGAT (A) or gephyrin (B) in hippocampal neurons. Scale bar, 10 μ m.

(C) Endogenous Shisa7 colocalized with gephyrin in hippocampal neurons. Scale bar, 10 μ m. (D) Stimulated emission depletion microscopy (STED) with the line profile analysis showed that Shisa7 and GABA_AR subunits colocalized in HEK cells ($n = 164$ puncta, $n = 8$ cells) (top) and in neurons ($n = 107$ puncta, $n = 8$ neurons) (bottom). The s prefix indicates surface. Scale bar, 60 nm. (E) Single-cell KO of Shisa7 strongly reduced mIPSC frequency but not amplitude in CA1 pyramidal cells ($n = 15$ for both conditions; t test). Kolmogorov-Smirnov test was used for cumulative distributions. Scale bars, 20 pA and 1 s. (F) Dual recording showed that Shisa7 sgRNA^{#1} strongly reduced IPSCs in CA1 pyramidal cells ($n = 16$; paired t test). (G) Anatomical reconstructions illustrating a representative CA1 pyramidal cell (red) innervated by a CCKBC (blue) and a CCKDTI (green), used for paired recordings. Scale bar, 100 μ m. S.O., stratum oriens; S.P., stratum pyramidale; S.R., stratum radiatum. (H) (Top) Sample averaged traces of CCKBC presynaptic action potentials and corresponding postsynaptic pyramidal neuron uIPSCs evoked by presynaptic stimuli in Shisa7 WT (black) and KO (red) hippocampal slices with individual sweeps shown in gray. Bars: 100 ms/100 pA or 50 mV. (Bottom) Boxed regions of trains (dashed lines in top traces) shown on an expanded time scale. Bar: 12.5 ms. On the right are summary plots of uIPSC amplitudes and potency (CCKBCs: WT $n = 20$, KO $n = 23$; CCKDTIs: WT $n = 12$, KO $n = 6$; Mann-Whitney U test). Error bars indicate SEM. **** $P < 0.0001$; * $P < 0.05$.



reduced surface expression levels of $\alpha 1$, $\alpha 2$, and $\gamma 2$ GABA_AR subunits in hippocampal neurons (Fig. 2D and fig. S12, A to I).

The total expression levels of $\alpha 2$ and $\alpha 3$ were significantly reduced in Shisa7 KO hippocampal lysates (fig. S13A). Moreover, $\alpha 1$, -2, and -3; $\beta 2$ and -3; and $\gamma 2$ were significantly decreased in postsynaptic fractions prepared from Shisa7 KO mice (Fig. 2E), indicating reduced synaptic GABA_ARs in Shisa7 KO neurons. Finally, post-embedding immunogold electron microscopy confirmed that both $\alpha 1$ and $\alpha 2$ were strongly decreased at morphologically identifiable somatic symmetric synapses in hippocampal

CA1 region in Shisa7 KO mice (Fig. 2F and fig. S13B).

Analysis of mIPSCs revealed a significantly slower decay in CA1 pyramidal neurons expressing Shisa7 sgRNA^{#1} and those from Shisa7 KO mice compared with control cells (Fig. 3A and fig. S14A). The decay time constant of CCKBC-mediated uIPSCs was also significantly increased in Shisa7 KO CA1 pyramidal neurons (fig. S14B), suggesting that Shisa7 may speed up GABA_AR decay kinetics at synapses. Indeed, at artificial GABAergic synapses formed between hippocampal cultures and HEK cells expressing GABA_ARs (Fig. 3B), Shisa7 signif-

icantly decreased the decay time constant of spontaneous IPSCs (sIPSCs) recorded in cells expressing $\alpha 1\beta 2\gamma 2$ or $\alpha 2\beta 3\gamma 2$ (Fig. 3C and fig. S14D). Notably, sIPSC amplitude was significantly higher in cells expressing $\alpha 2\beta 3\gamma 2$, but not $\alpha 1\beta 2\gamma 2$, with Shisa7 (fig. S14, C and E), consistent with the data presented in fig. S11D. In outside-out patches from HEK cells expressing $\alpha 1\beta 2\gamma 2$ or $\alpha 2\beta 3\gamma 2$, Shisa7 decreased the weighted time constant of deactivation as well as both fast (τ_{fast}) and slow (τ_{slow}) components of deactivation (Fig. 3D and fig. S14, F and G). However, Shisa7 did not change desensitization of $\alpha 1\beta 2\gamma 2$ (fig. S14H).

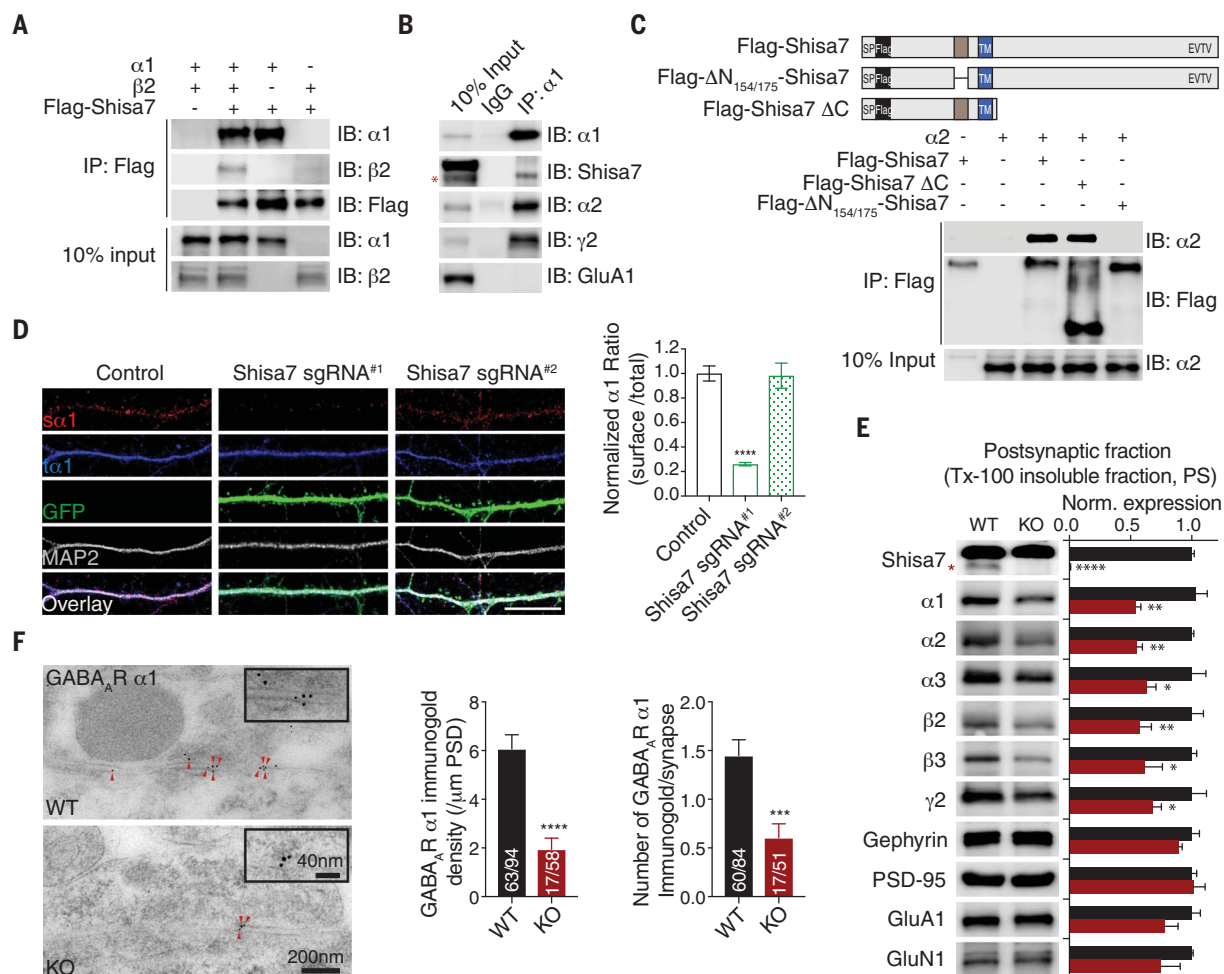


Fig. 2. Shisa7 binds GABA_ARs through a distinctive N-terminal domain and controls synaptic abundance of GABA_ARs. (A and B) The α1 subunit coimmunoprecipitated with Shisa7 in HEK cells (A) and in hippocampal lysates (B). IB, immunoblot; IgG, immunoglobulin G. (C) Schematic of Flag-Shisa7, Flag-ΔN_{154/175}-Shisa7, and Flag-Shisa7ΔC. SP, signal peptide; TM, transmembrane domain. In HEK cells, the α2 subunit coimmunoprecipitated with Flag-Shisa7 and Flag-Shisa7ΔC but not Flag-ΔN_{154/175}-Shisa7. (D) Expression of Shisa7 sgRNA^{#1}, but not sgRNA^{#2}, in hippocampal neurons reduced the ratio of surface α1 (sa1) and total α1 (ta1). Control *n* = 39; sgRNA^{#1} *n* = 21; sgRNA^{#2} *n* = 18. A one-way analysis of variance (ANOVA) was used. Scale bar, 10 μm. (E) Protein

expression in postsynaptic fractions (PS) from Shisa7 WT and KO mice (*n* = 5 for both conditions; one-way ANOVA was used). Tx-100, Triton X-100. (F) Postembedding immunogold labeling of the α1 subunit in the hippocampal CA1 region showed that Shisa7 KO significantly reduced the density of α1 at the inhibitory postsynaptic density (PSD). Data for α1 density (control *n* = 63/94, KO *n* = 17/58) and data for immunogold labeling per synapse (control *n* = 60/84, KO *n* = 17/51) were evaluated with a *t* test. Scale bar, 200 nm (low-magnification images) or 40 nm (high-magnification images). The red asterisk indicates the Shisa7 band. Error bars indicate SEM. *****P* < 0.0001; ****P* < 0.001; ***P* < 0.01; **P* < 0.05.

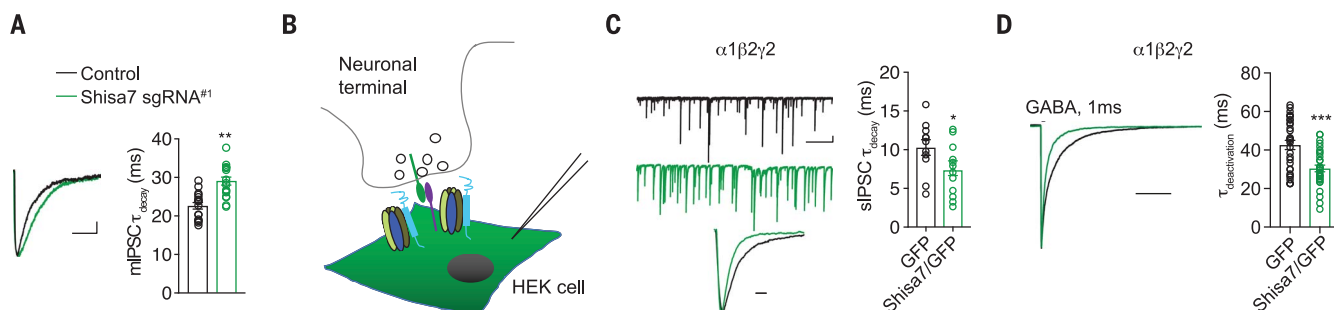


Fig. 3. Shisa7 modulates kinetics of GABAergic transmission and GABA_ARs. (A) The mIPSC decay time constant was significantly increased in CA1 neurons expressing sgRNA^{#1} (*n* = 14 for both conditions; *t* test). Scale bar, 20 ms, 2 pA. (B) Schematic of coculture of hippocampal neurons with HEK cells. (C) Shisa7 significantly reduced the decay time constant of sIPSCs mediated by α1β2γ2

(*n* = 13 for both conditions; *t* test) in HEK cells in coculture. Scale bar, 20 pA, 1 min. (Bottom) Peak-normalized sample traces. Scale bar, 10 ms. GFP, green fluorescent protein. (D) Coexpression with Shisa7 significantly reduced the weighted time constant of deactivation of α1β2γ2 in HEK cells (*n* = 31 for both conditions; *t* test). (Left) Peak-normalized sample traces. Scale bar, 50 ms. ****P* < 0.001; ***P* < 0.01; **P* < 0.05.

Diazepam (DZ) is a positive allosteric modulator of GABA_ARs (7–9). In HEK cells, while DZ potentiated nonsaturating GABA-evoked currents of $\alpha 2\beta 3\gamma 2$ receptors, it enhanced currents of $\alpha 2\beta 3\gamma 2$ /Shisa7 complexes to a greater degree (Fig. 4A and fig. S15A). None of the other known or putative GABA_AR interactors recently identified in GABA_AR proteomes (14–20) increased the potentiation of GABA responses (Fig. 4A and fig. S15B). Shisa7 significantly increased the potentiation of GABA responses in cells expressing $\alpha 1\beta 3\gamma 2$, but not cells expressing $\alpha 1\beta 2\gamma 2$ (fig. S16, A to D). Conversely, in Shisa7 KO hippocampal neurons, the potentiating effect of DZ on GABA-evoked currents was strongly diminished (Fig. 4B and fig. S16E). Similarly, DZ-induced enhancement of sIPSCs was greater in WT CA1 neurons than in Shisa7 KO neurons (fig. S16, F and G).

General behavioral characterizations revealed no obvious differences between WT and Shisa7 KO mice in novel object recognition, social interaction, and marble-burying tests (fig. S17, A and B). However, the action of DZ was severely blunted in Shisa7 KO mice (Fig. 4, C to G). An anxiolytic dose of DZ [1.5 mg per kilogram of body weight (mg/kg), administered intraperitoneally (i.p.)] significantly increased the number of elevated plus maze open arm entries and the time spent in the open arm for WT mice but not for Shisa7 KO mice (Fig. 4, C and D). In addition, DZ strongly reduced motor activity in WT mice but its effect was diminished in Shisa7 KO mice, suggesting a reduced sedative effect of DZ in KO mice (fig. S18A). The ability of DZ to induce the loss-of-righting reflex (LORR) in Shisa7 KO mice also was strongly decreased (Fig. 4E). At the hypnotic dose used (50 mg/kg, i.p.), DZ consistently produced LORR in all WT mice, whereas only ~60 to 70% of Shisa7 KO mice showed the LORR after DZ administration (Fig. 4F and fig. S18B). In the responding population of Shisa7 KO mice, LORR induced by DZ (50 mg/kg, i.p.) was severely compromised, as indicated by substantially increased latency to LORR combined with a markedly shortened duration of LORR (Fig. 4G, fig. S18B, and movies S1 and S2).

We identified Shisa7 as a GABA_AR auxiliary subunit—i.e., one that possibly interacts directly with GABA_ARs, regulates GABA_AR trafficking to synapses, and modulates the channel kinetics and pharmacology (see the supplementary text for further discussion). Functionally, Shisa7 plays a crucial role in controlling GABAergic transmission. Genetic inactivation of Shisa7 led to a strong reduction of synaptic GABA_ARs as well as GABAergic transmission in hippocampal neurons. In addition, Shisa7 shapes inhibitory synaptic responses by speeding up channel decay kinetics. Several membrane molecules (i.e., LHFPL4/GARLH4 and Clptm1) regulate syn-

aptic anchorage and trafficking of GABA_ARs, although these molecules appear not to modulate GABA_AR kinetics (16–19). Our finding that Shisa7 regulates both trafficking and kinetics of the Cl[−] channel indicates that functional properties of GABA_ARs are regulated by an auxiliary molecule, in addition to the pore-forming subunits, providing a new dimension for understanding and reanalysis of GABA_AR function and pharmacology.

Shisa7 enhances DZ-induced potentiation of both $\alpha 1$ - and $\alpha 2$ -containing GABA_ARs in heter-

ologous cells. Correspondingly, disruption of Shisa7-mediated GABA_AR regulation in mice significantly reduced DZ actions at cellular and network behavioral levels. Previous studies have demonstrated that distinct GABA_AR subtypes mediate sedative or anxiolytic-like effects of BDZs (21–24). This indicates that Shisa7 may influence GABA_AR subtype-specific therapeutic effects of BDZs in vivo. Mechanistically, Shisa7 may modulate DZ effects through regulation of BDZ-sensitive GABA_AR trafficking to the cell surface and/or increasing DZ efficacy at cell

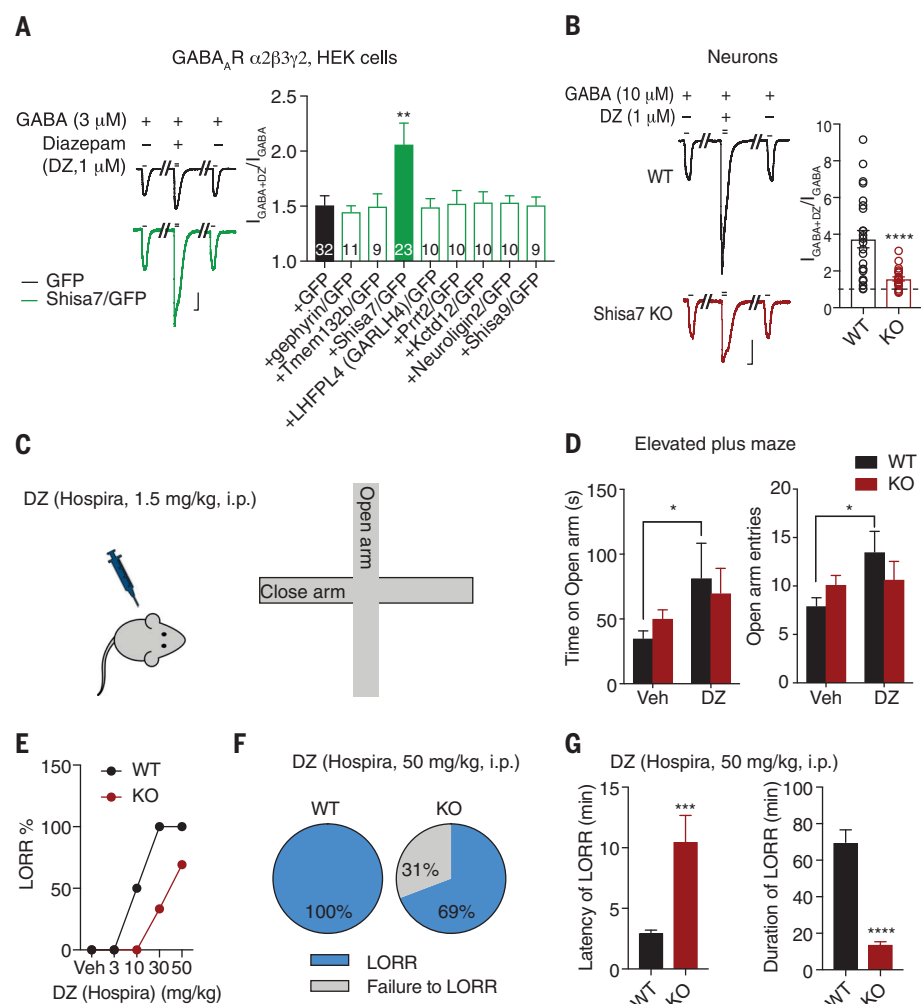


Fig. 4. Shisa7 KO diminishes DZ effects in vitro and in vivo. (A) Shisa7, but not other molecules, potentiated the DZ effect on GABA response of $\alpha 2\beta 3\gamma 2$ in HEK cells (one-way ANOVA). Scale bar, 200 pA, 2 s. (B) Shisa7 KO significantly reduced DZ-induced potentiation of GABA-evoked whole-cell currents in cultured neurons (WT $n = 24$; KO $n = 23$; t test). Scale bar, 500 pA, 2 s. (C and D) DZ (1.5 mg/kg) increased the time spent on open arm and the number of open arm entries in WT mice but not Shisa7 KO mice. Veh, vehicle. (Treatment groups: Veh WT $n = 14$ and KO $n = 21$; DZ WT $n = 10$ and KO $n = 15$.) Time: treatment [$F_{1,56} = 5.113$, $P < 0.05$]; group [$F_{1,56} = 0.016$, $P > 0.05$]; treatment $\times$ group [$F_{1,56} = 0.845$, $P > 0.05$]; entries: treatment [$F_{1,56} = 4.501$, $P < 0.05$]; group [$F_{1,56} = 0.035$, $P > 0.05$]; treatment $\times$ group [$F_{1,56} = 3.101$, $P > 0.05$]; two-way ANOVA. (E) Dose-response relationship of DZ in LORR [Veh $n = 6$ for both conditions; DZ (3 mg/kg) $n = 6$ for both conditions; DZ (10 mg/kg) WT $n = 6$ for both conditions; DZ (30 mg/kg) WT $n = 7$, KO $n = 6$; DZ (50 mg/kg) WT $n = 13$ for both conditions]. (F) DZ at the hypnotic dose produced LORR in 100% of WT mice ($n = 13$), but only 69% of KO mice (9 of 13 mice). (G) Shisa7 KO significantly prolonged latency to LORR and shortened duration of LORR after DZ administration (WT $n = 13$, KO $n = 9$; t test). Error bars indicate SEM. **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

surface GABA_ARs. Our identification of Shisa7 as an auxiliary subunit of GABA_ARs capable of modulating DZ actions in vivo highlights a molecular target that can be leveraged for future refinement of psychopharmacological GABA_AR therapeutics, an emerging drug discovery strategy that has shown promise in targeting auxiliary subunits of other ion channels (25).

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C.J.M., Y.-X.W., R.S.P., L.-G.W., and C.L.) and NHMRC 1058542 and 1120947, Australia (to J.W.L.). The Advanced Imaging Core code is ZIC DC000081. **Author contributions:** W.H. and W.L. designed the project, and W.L. conceived and supervised the project. W.H. performed molecular, immunocytochemical, imaging, biochemical, and electrophysiological experiments in HEK cells and neurons and behavioral experiments. J.L., S.P., and T.L. performed some of the immunostaining. K.A.P. and C.J.M. designed and performed paired recordings between pyramidal neurons and interneurons. X.C. and J.W.L. performed deactivation and desensitization experiments in HEK cells. W.H., Y.-X.W., and R.S.P. performed electron microscopy experiments. W.H., L.G., and L.-G.W. performed STED imaging. K.W. and D.C. performed some recordings. C.L. developed the Shisa7 KO mouse line. W.H. and W.L. wrote the manuscript with contributions from all other authors. **Competing interests:** None declared. **Data and materials availability:** All data are available in the manuscript and supplementary materials.

SUPPLEMENTARY MATERIALS

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NEUROSCIENCE

Control of aversion by glycine-gated GluN1/GluN3A NMDA receptors in the adult medial habenula

Y. Otsu^{1*†}, E. Darceq^{2†}, K. Pietrajtis^{3†}, F. Mátyás^{4,5,6}, E. Schwartz³, T. Bessaih³, S. Abi Gerges³, C. V. Rousseau^{1†}, T. Grand¹, S. Dieudonné¹, P. Paoletti¹, L. Acsády⁴, C. Agulhon⁷, B. L. Kieffer², M. A. Diana^{3§}

The unconventional *N*-methyl-D-aspartate (NMDA) receptor subunits GluN3A and GluN3B can, when associated with the other glycine-binding subunit GluN1, generate excitatory conductances purely activated by glycine. However, functional GluN1/GluN3 receptors have not been identified in native adult tissues. We discovered that GluN1/GluN3A receptors are operational in neurons of the mouse adult medial habenula (MHb), an epithalamic area controlling aversive physiological states. In the absence of glycinergic neuronal specializations in the MHb, glial cells tuned neuronal activity via GluN1/GluN3A receptors. Reducing GluN1/GluN3A receptor levels in the MHb prevented place-aversion conditioning. Our study extends the physiological and behavioral implications of glycine by demonstrating its control of negatively valued emotional associations via excitatory glycinergic NMDA receptors.

Glycine is a major inhibitory neurotransmitter of the central nervous system, which acts via anion-permeable receptors. It is also a well-characterized coagonist of excitatory *N*-methyl-D-aspartate receptors (NMDARs) via GluN1 subunits (1). In addition, glycine binds the unconventional

NMDAR subunits GluN3A and GluN3B, which generate atypical glutamate-activated triheteromeric NMDARs with GluN1 and GluN2 subunits (2), as well as glycine-gated diheteromeric excitatory complexes with GluN1 (3). GluN3B is appreciably expressed only in caudal areas (4). GluN3A expression is broader

but assumed to be limited to early development (2). Mainly examined in recombinant systems (5), native GluN1/GluN3A receptors (GluN1/GluN3ARs) have been identified in the juvenile hippocampus (6) but never in adult neurons.

We found strong immunohistochemical expression of GluN3A subunits in the ventral subdivision of the medial habenula (MHb) of adult mice (Fig. 1A) (7), an area that mediates aversive behaviors (8–12). We investigated GluN3A-immunostained sections with electron microscopy (EM) to identify the ultrastructural localization of the subunit. Frequently in juxtaposition with glial structures, all the 3,3-diaminobenzidine (DAB)-labeled profiles were identified as postsynaptic dendrites and somata ($n = 92$) (Fig. 1B). Supported by further confocal microscopy results (fig. S1), these data demonstrated that GluN3A subunits were abundantly expressed in adult MHb neurons.

We next examined whether GluN3A subunits formed operational GluN1/GluN2/GluN3ARs. These receptors show reduced rectification compared with GluN1/GluN2 NMDARs (2). We thus examined the current-voltage (*I*-*V*) curves of synaptic (13) and puff-evoked NMDAR currents in MHb neurons from wild-type (WT)

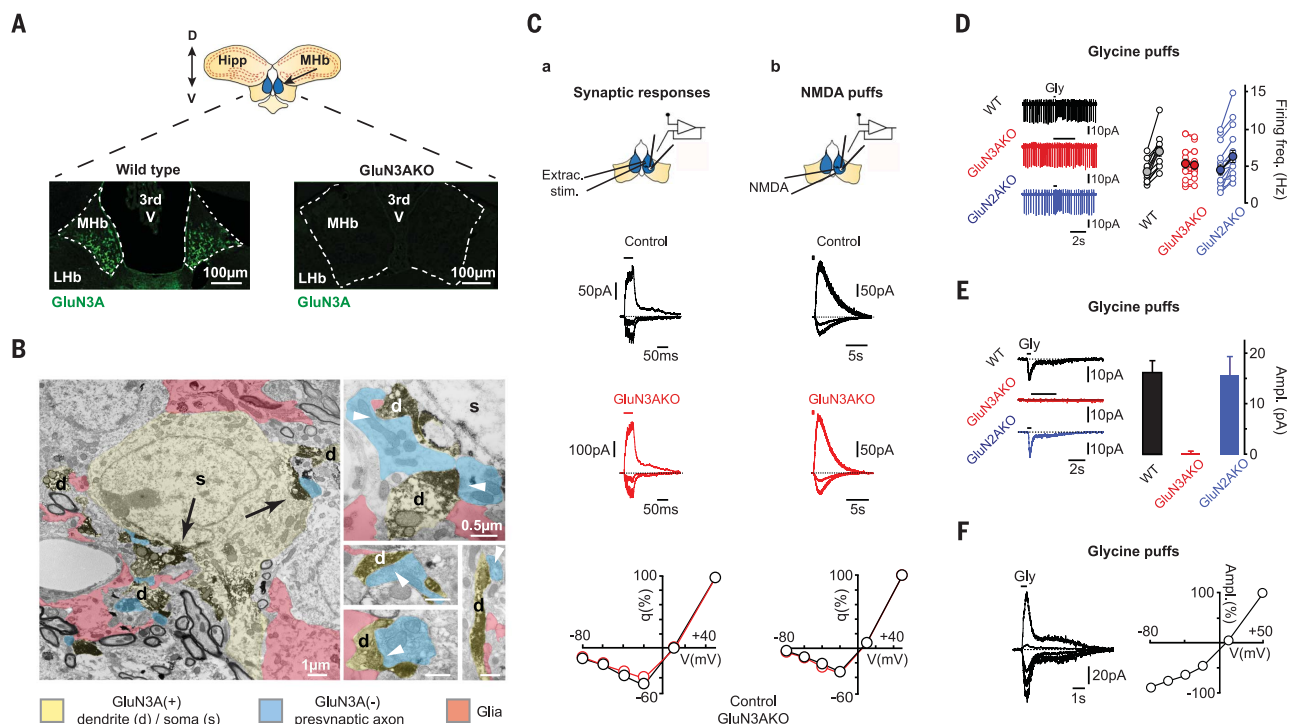


Fig. 1. Functional GluN1/GluN3ARs in the adult MHb. Both (A) confocal and (B) electron microscopy demonstrated that GluN3A subunits are expressed in the ventral MHb. DAB EM staining [black deposits in (B)] was detected in dendrites (yellow), close to glial extensions (red), rarely in somas (black arrows), but never in axons (blue; white arrowheads highlight presynaptic specializations). Hipp, hippocampus; LHb, lateral habenula; 3rd V, third ventricle. (C) GluN1/GluN2/GluN3ARs are not functional in the

MHb. No difference was found in the rectification of NMDAR currents in WT (black) and GluN3AKO (red) mice, elicited by extracellular stimulation (a) or pressure-ejected NMDA (b). (D) Glycine puffs increased firing rates in MHb cells from control and GluN2AKO mice, but not from GluN3AKO animals. (E and F) Similarly to heterologous GluN1/GluN3R currents, glycine induced outwardly rectifying, rapidly rising and inactivating currents in control and GluN2AKO, but not GluN3AKO, neurons. Data are illustrated as averages $\pm$ SEM.

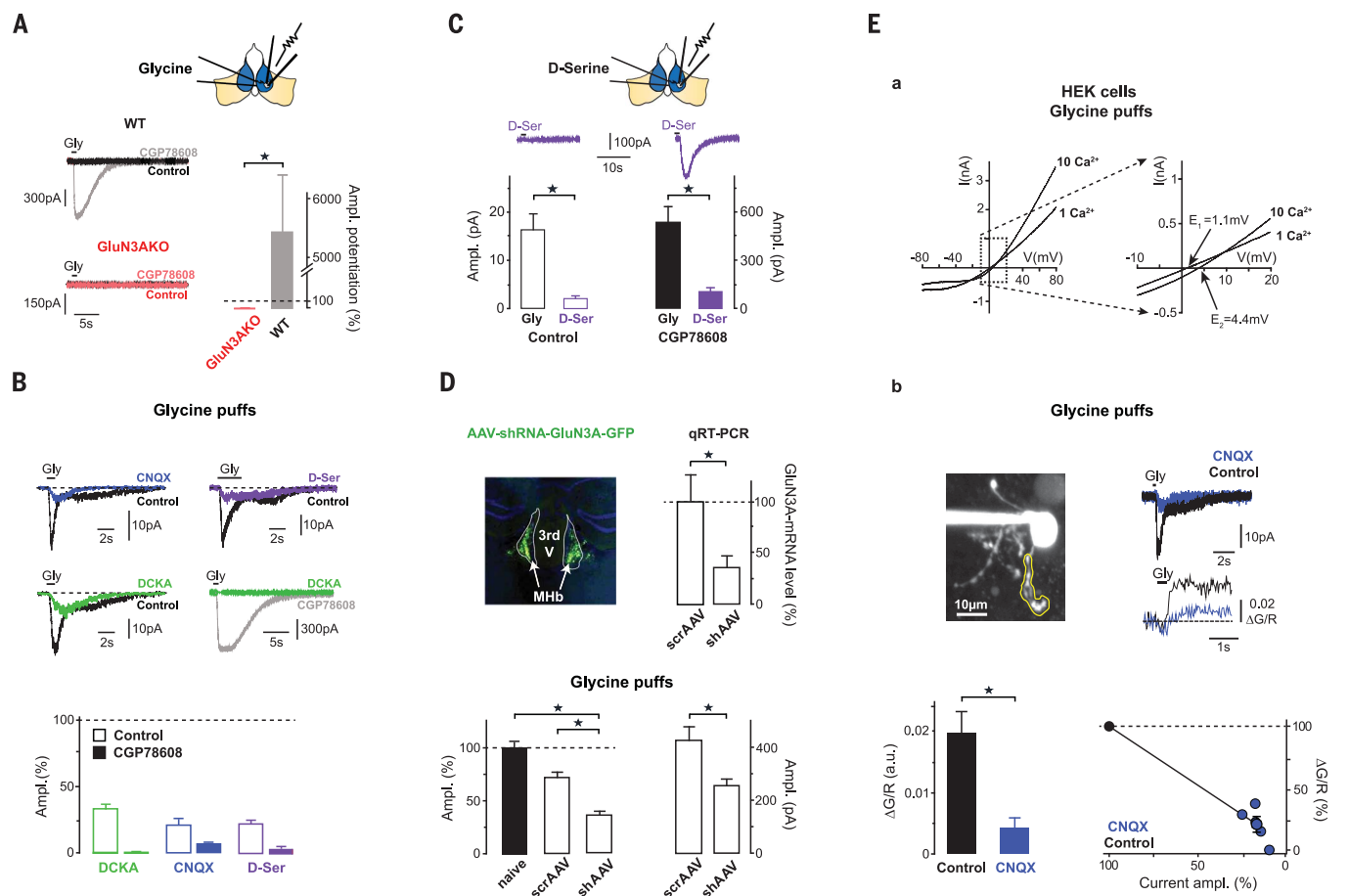


Fig. 2. Properties of GluN1/GluN3Rs in the MHB. (A) GluN1 subunit block with CGP78608 potentiated glycine-elicited currents. (B) Bath applications of CNQX (blue), 5,7-DCKA (green), and D-serine (purple) antagonized control and potentiated GluN1/GluN3AR currents. (C) Pressure-ejected D-serine had only partial agonist effects on GluN1/GluN3ARs. (D) Viral expression in the MHB of a GluN3A-targeting shRNA (AAV-shRNA-GluN3A-GFP and shRNA) led to reduction of GluN3A mRNA levels, and of glycine-induced currents with respect to scrambled RNA-expressing mice (scrAAV). Percentage values and absolute amplitudes

are illustrated in the lower graphs. (E) GluN1/GluN3ARs are Ca²⁺ permeable. Changing extracellular Ca²⁺ concentrations led to a significant reversal potential shift [(a), highlighted on the right] in heterologous systems expressing GluN3A and point-mutated, glycine-insensitive GluN1 subunits. In the presence of synaptic receptor antagonists and Ca²⁺ channel blockers, two-photon imaging revealed glycine-elicited Ca²⁺ transients in ex vivo MHB neurons (b). ΔG/R, ratio between green fluorescence increases and basal red fluorescence. Stars represent statistically significant differences. Data are illustrated as averages ± SEM.

and GluN3A knock-out (GluN3AKO) (14) mice, in which excitatory transmission to the MHB is not modified (fig. S2). No significant differences were found (Fig. 1C, a and b), suggesting the absence of functional receptors.

To investigate whether glycine-activated GluN1/GluN3ARs were operational, glycine (1 or 10 mM) was pressure-ejected while recording spontaneous firing activity (13) in the loose cell-attached configuration (LCA). In all tested WT neurons, glycine puffs (300 to 500 ms) potently increased firing in the presence of D-2-amino-5-phosphonovalerate (D-APV)

and strychnine (Fig. 1D). This excitatory effect was not attributable to conventional NMDARs, because it was present in GluN2AKO mice (Fig. 1D), where GluN1/GluN2 NMDARs are nearly undetectable (13). The effect on firing activity was absent in GluN3AKO mice (Fig. 1D), supporting the presence of GluN1/GluN3ARs in the MHB.

Glycine puffs induced rapidly rising and inactivating inward currents in voltage clamp recordings of both WT and GluN2AKO ex vivo MHB neurons performed in the presence of D-APV and strychnine. The currents could not

be elicited in GluN3AKO mice (Fig. 1E). As in recombinant systems, the glycine-evoked currents showed slight outward rectification (Fig. 1F).

We investigated the pharmacology of the glycine-induced currents. In recombinant systems, occupation of the higher-affinity GluN3A site activates the receptor, whereas glycine binding to GluN1 entrains rapid desensitization (5, 15). We recently discovered that the GluN1 antagonist [(1S)-1-[[[7-Bromo-1,2,3,4-tetrahydro-2,3-dioxo-5-quinoxaliny]methyl]amino]ethyl] phosphonic acid (CGP78608) enhanced receptor

¹Institut de Biologie de l'École Normale Supérieure (IBENS), INSERM U1024, CNRS UMR8197, École Normale Supérieure, Université PSL, 75005 Paris, France. ²Department of Psychiatry, School of Medicine, Douglas Hospital Research Center, McGill University, Montreal, QC H4H 1R3, Canada. ³Sorbonne Université, CNRS, INSERM, Neurosciences Paris Seine – Institut de Biologie Paris Seine (NPS-IBPS), 75005 Paris, France. ⁴Laboratory of Thalamus Research, Institute of Experimental Medicine, Hungarian Academy of Sciences, 1083 Budapest, Hungary. ⁵Research Centre for Natural Sciences Institute of Cognitive Neuroscience and Psychology, 1117 Budapest, Hungary. ⁶Department of Anatomy and Histology, University of Veterinary Medicine, 1078 Budapest, Hungary. ⁷Integrative Neuroscience and Cognition Center, CNRS UMR8002, Glia-Glia and Glia-Neuron Interactions Group, Paris Descartes University, 75006 Paris, France.

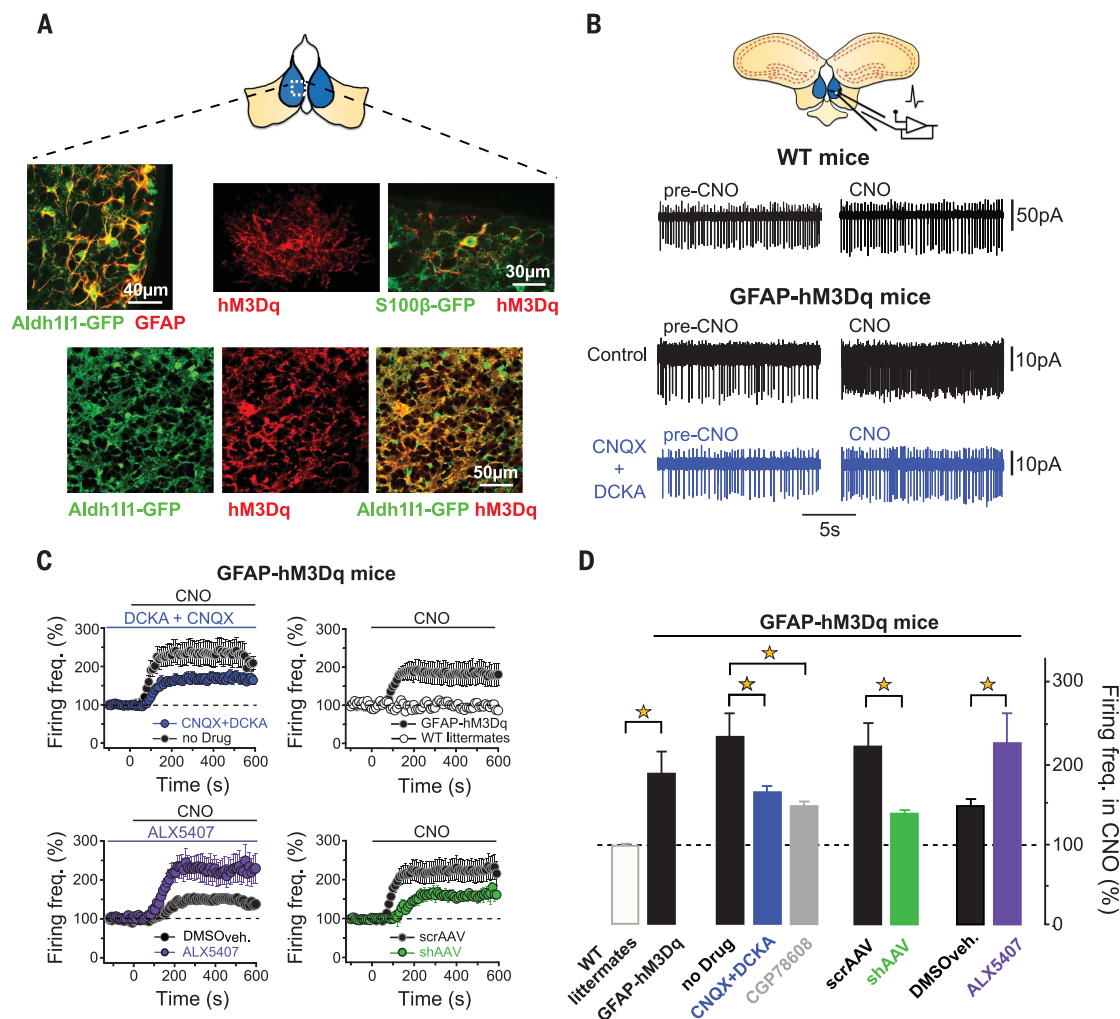
*Present address: Pain Management Research Institute, Kolling Institute of Medical Research, Northern Clinical School, The University of Sydney and Royal North Shore Hospital, St. Leonards, NSW 2065, Australia.

†These authors contributed equally to this work. ‡Present address: Neural Circuit Dynamics and Decision Making, Pasteur Institute, 75015 Paris, France.

§Corresponding author. Email: marco.diana@upmc.fr

Fig. 3. Glial activation in GFAP-hM3Dq mice increases neuronal excitability via GluN1/GluN3ARs.

(A) The specific expression of hM3Dq receptors in MHb glia was demonstrated by the colocalization of GFAP and GFP in aldehyde dehydrogenase-1 family member 11-green fluorescent protein (Aldh1l1-GFP) mice, and of hM3DqRs and GFP in GFAP-hM3Dq::Aldh1l1-GFP and GFAP-hM3Dq::S100 β -GFP mice. (B) CNO applications increased spontaneous firing rates of MHb neurons. Representative traces preceding and following CNO are shown for specified experiments. The average time courses (C) and quantifications (D) of the CNO effects are depicted for all the experiments. The smaller increase of the firing rate in CGP78608 than in control likely derived from reduced affinity for glycine in the presence of the drug (6). DMSO veh., dimethyl sulfoxide vehicle. Stars represent statistically significant differences. Data are illustrated as averages $\pm$ SEM.



responses by reducing desensitization (6). We found that CGP78608 (1 μ M) potentiated and prolonged the responses in WT but not GluN3AKO mice (Fig. 2A). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), 5,7-Dichlorokynurenic acid (5,7-DCKA), and D-serine inhibit the currents produced by GluN1/GluN3Rs (3, 5, 6, 15). These drugs also potently inhibited the responses to glycine in MHb neurons (Fig. 2B). Direct applications of D-serine increased neuronal excitability (fig. S4) and elicited currents (Fig. 2C) to a lesser extent than glycine (3, 15). With the exception of zinc, which had no effects (16) (fig. S5), the pharmacological profile of the native currents thus mirrored that of recombinant GluN1/GluN3Rs.

We injected the MHb with a short hairpin ribonucleic acid (shRNA)-expressing adeno-associated virus (AAV) targeting the GluN3A subunit (AAV5-shRNA-GluN3A) (17). The virus efficiently reduced both the messenger RNA levels of GluN3A in the MHb (Fig. 2D) and the amplitude of the glycine-induced currents compared with scrambled RNA (AAV5-scrRNA-GluN3A)-expressing mice (Fig. 2D).

The permeability of GluN1/GluN3ARs to calcium (Ca^{2+}) remains uncertain (3, 18). We examined this question by first analyzing in human embryonic kidney (HEK) cells a GluN1/GluN3AR variant with potentiated glycine responses (GluN1-F484A/GluN3A) (5, 6). Increasing the extracellular Ca^{2+} concentration led to a shift of the reversal potential of the responses to glycine (Fig. 2E, a), which demonstrated significant Ca^{2+} permeability, although smaller than for WT GluN1/GluN2A receptors (fig. S6).

Using two-photon microscopy in the presence of synaptic and Ca^{2+} channel blockers, we could detect glycine-evoked Ca^{2+} transients originating from GluN1/GluN3ARs in ex vivo MHb cells (Fig. 2E, b). We concluded that native GluN1/GluN3A receptors were also permeable to Ca^{2+} .

We then searched for physiological sources of glycine. We had previously found that the MHb was completely devoid of glycinergic neuronal specializations (fig. S1) (19). Thus, we tested whether glial cells could control extracellular glycine levels in mice expressing the excitatory DREADD (designer receptor exclu-

sively activated by designer drugs) hM3Dq under the control of the promoter of the glial fibrillary protein (GFAP). In these mice, hM3Dq receptor (hM3DqR) activation triggers Ca^{2+} elevations specifically in glial cells (20). We confirmed that hM3DqR expression was exclusively glial (Fig. 3A and fig. S7).

To determine whether GluN1/GluN3ARs mediated glia-neuron interactions in the MHb, we recorded ex vivo spontaneous firing activity in GFAP-hM3Dq mice (Fig. 3). Application of the hM3DqR agonist clozapine-N-oxide (CNO; 10 μ M), produced rapid firing rate increases. Preincubation with 5,7-DCKA and CNQX strongly reduced the effect of CNO, suggesting a substantial contribution from GluN1/GluN3ARs (Fig. 3). CGP78608 application significantly augmented basal firing rates (fig. S3), indicating that ambient glycine may suffice to bind GluN3A. The potentiation of the firing rate triggered by CNO was instead reduced by CGP78608 preincubations.

Furthermore, the effect of CNO was significantly smaller in GFAP-hM3Dq mice injected with the AAV5-shRNA-GluN3A-GFP than with AAV5-scrRNA-GluN3A-GFP virus (GFP, green

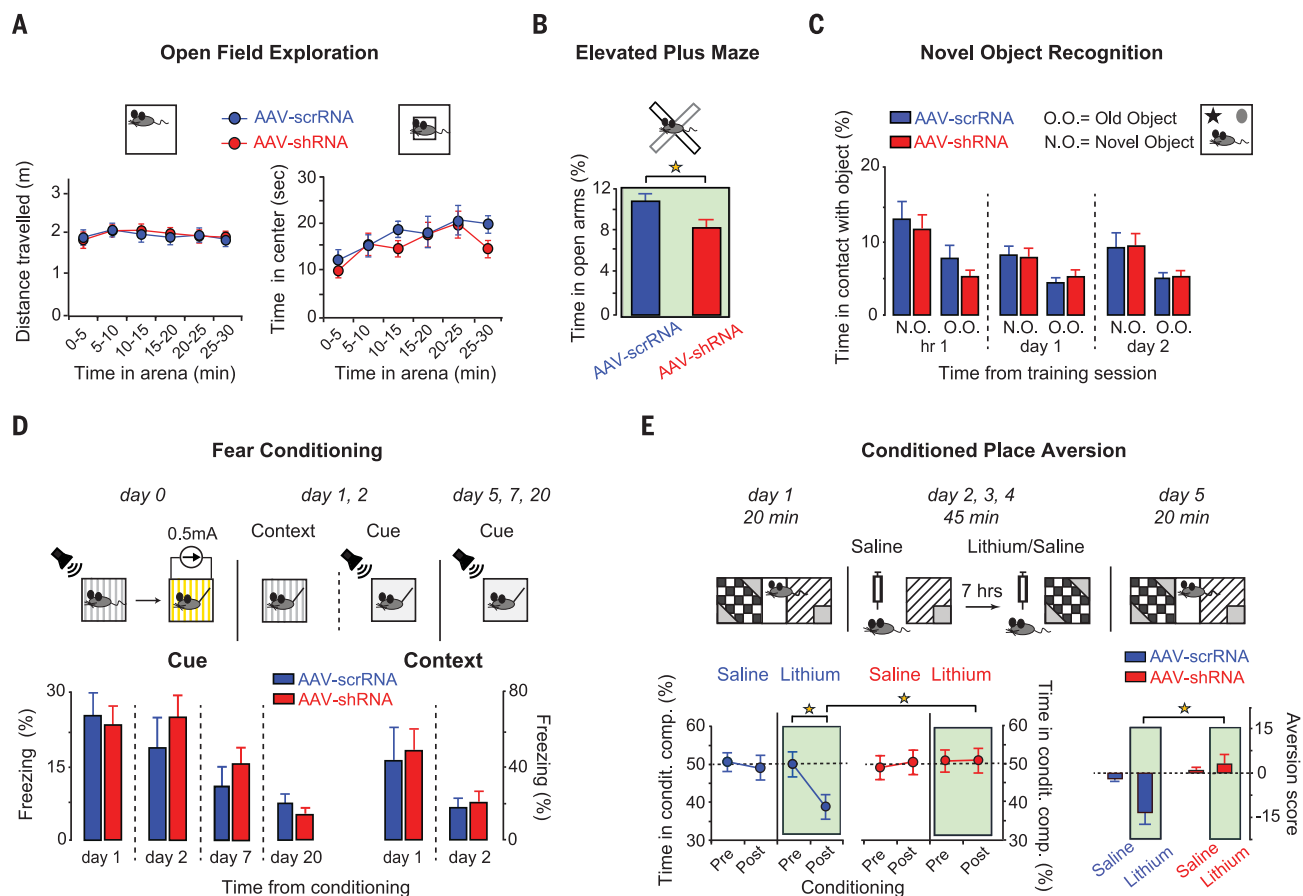


Fig. 4. MHb GluN1/GluN3ARs control place-aversion conditioning. (A) No difference was detected between AAV-shRNA-GluN3A- and AAV-scrRNA-GluN3A-injected mice in the distance travelled in an open field and in the time spent in its center. (B) Knocking down MHb GluN3A expression was mildly anxiogenic as indicated by the shorter time spent by GluN3A-deficient mice in the open arms of an elevated plus maze. Object exploration times and cue- and context-induced freezing were similar in (C) novel object recognition and (D) fear conditioning tests, respectively. (E) After lithium conditioning, AAV-shRNA-GluN3A-injected mice developed no aversion for the malaise-associated compartment in contrast to scrRNA-expressing animals. Data are illustrated as averages $\pm$ SEM.

fluorescent protein) (Fig. 3). In contrast to GlyT2 (fig. S1), we found expression in MHb glia of the membrane glycine transporter GlyT1 (fig. S8), which can contribute to glial glycine accumulation (21). In the presence of the GlyT1-specific blocker *N*-[(3*R*)-3-([1,1'-Biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-*N*-methylglycine (ALX5407), CNO applications increased firing rates to greater values than in control (Fig. 3, C and D), likely because of greater buildup of extracellular glycine levels. Together, these experiments suggested that glial cells potentiated neuronal activity via activation of GluN1/GluN3ARs.

Finally, we examined whether GluN1/GluN3ARs in the adult MHb were behaviorally relevant in mice injected with either the AAV5-shRNA-GluN3A-GFP or the AAV5-scrRNA-GluN3A-GFP virus (fig. S9). No differences were detected in locomotor and exploratory activity in an open field arena (Fig. 4A). In contrast, the test mice spent significantly less time in the open arms of an elevated place maze, suggesting that reduced GluN1/GluN3AR

levels in the MHb were mildly anxiogenic (Fig. 4, A and B). GluN1/GluN3ARs in the MHb did not modulate learning and memory capabilities, because test and control mice scored similarly in a novel object recognition test (Fig. 4C).

The MHb can contribute to the development of negatively valued emotional states. We thus examined the behavioral outcomes of a fear conditioning protocol (Fig. 4D). We found no difference between AAV5-shRNA- and AAV5-scrRNA-injected mice in the freezing time elicited either by the cued or the contextual stimulus (Fig. 4D).

Finally, we examined lithium-induced conditioned place aversion, a paradigm that relies on intact MHb function (22, 23). In contrast to AAV5-scrRNA animals, lithium-treated AAV5-shRNA mice did not develop aversion for the conditioned compartment (Fig. 4E). Overall, these results indicate that impaired GluN3A signaling in the MHb alters the capability of associating negatively valued external conditions with internal aversive states.

In addition to its ubiquitous role as an inhibitory neurotransmitter, glycine can exert excitatory actions via unconventional GluN1/GluN3A NMDARs. Our study unveils a functional role of this aspect of glycine physiology by demonstrating that full expression of GluN1/GluN3ARs in the MHb is mandatory for modifying the internal emotional landscape in response to specific environmental challenges.

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F.M. performed electron microscopy under supervision of L.A. E.D. performed the behavioral tests under the supervision of B.L.K., with M.A.D. and K.P. T.G. performed the HEK experiments under supervision of P.P. S.D. contributed equipment. C.A. provided transgenic mice. M.A.D. wrote the manuscript, which was edited by all authors. **Competing interests:** The authors declare no competing interests. **Data and materials availability:** Data values and statistics are included as supplementary materials.

SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/366/6462/250/suppl/DC1
Materials and Methods
Figs. S1 to S9
Table S1
References (24–39)

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ECOSYSTEM SERVICES

Global modeling of nature's contributions to people

Rebecca Chaplin-Kramer^{1,2*}, Richard P. Sharp¹, Charlotte Weil¹, Elena M. Bennett³, Unai Pascual^{4,5,6}, Katie K. Arkema^{1,7}, Kate A. Brauman², Benjamin P. Bryant^{1,8}, Anne D. Guerry^{1,7}, Nick M. Haddad⁹, Maïke Hamann^{2,10}, Perrine Hamel¹, Justin A. Johnson², Lisa Mandle¹, Henrique M. Pereira^{11,12,13}, Stephen Polasky¹⁴, Mary Ruckelshaus^{1,7}, M. Rebecca Shaw¹⁵, Jessica M. Silver^{1,7}, Adrian L. Vogt¹, Gretchen C. Daily^{1,16}

The magnitude and pace of global change demand rapid assessment of nature and its contributions to people. We present a fine-scale global modeling of current status and future scenarios for several contributions: water quality regulation, coastal risk reduction, and crop pollination. We find that where people's needs for nature are now greatest, nature's ability to meet those needs is declining. Up to 5 billion people face higher water pollution and insufficient pollination for nutrition under future scenarios of land use and climate change, particularly in Africa and South Asia. Hundreds of millions of people face heightened coastal risk across Africa, Eurasia, and the Americas. Continued loss of nature poses severe threats, yet these can be reduced 3- to 10-fold under a sustainable development scenario.

Evidence on how human actions cause environmental change, and how environmental change affects human well-being, can provide the basis for sound investments in nature benefitting people (1). The Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) was established to synthesize and advance science supporting such investments (2, 3) and recently completed its first Global Assessment compiling the overall status and trends of nature's contributions to people (4). However, spatially explicit modeling of many of these contributions, showing where nature matters most to people over global extents, has remained a major challenge (5).

Thanks to rapid improvements in spatial data, computation, and visualization, nature's contributions to people can now be quantified at policy-relevant scales in an accessible, inte-

grated, and globally consistent way. Here, we model three vital contributions spanning three realms of the biosphere (freshwater, coastal, and terrestrial) and representing contrasting biophysical processes: regulation of drinking-water quality through nitrogen retention, coastal risk reduction of hazards such as shoreline erosion and flooding, and wild pollination of crops for human nutrition.

The spatial dependence of the socioecological processes governing these contributions of nature to people require fine-scale data, differentiating them from the coarser-scale global mapping of contributions such as carbon se-

questration and storage (6). For example, a wetland downslope from a farm absorbs excess fertilizer; mangroves, coral reef, and coastal marshes close to vulnerable human communities confer storm protection; and a bee habitat within flight distance of crops enables wild pollination. To perform such fine-scale analysis over continental or global extents requires advanced computational capabilities. Previous global modeling approaches have disregarded spatial configuration of nature, in the case of coastal risk reduction (7), or have not accounted for the role of nature at all in retaining pollution (8, 9) or providing pollinators to farmland (10, 11) and thus cannot project how degradation of nature can affect human well-being. Our approach uses spatially explicit modeling to operationalize the IPBES conceptual framework for nature's contributions to people (12), which is achieved by enhancing and scaling up the InVEST (Integrated Valuation of Ecosystem Services and Trade-offs) modeling platform with free and open-source data and software (13) that have been widely deployed at regional to national scales (14).

We consider the dual dimensions of nature's contributions to people—(i) people's needs and (ii) nature's contributions (Fig. 1 and fig. S1)—and distinguish these contributions from ecosystem services (corresponding to “realized benefits” in Fig. 1) by considering the proportion of potential benefit provided by nature. A proportional representation is important to track differences or changes across space and

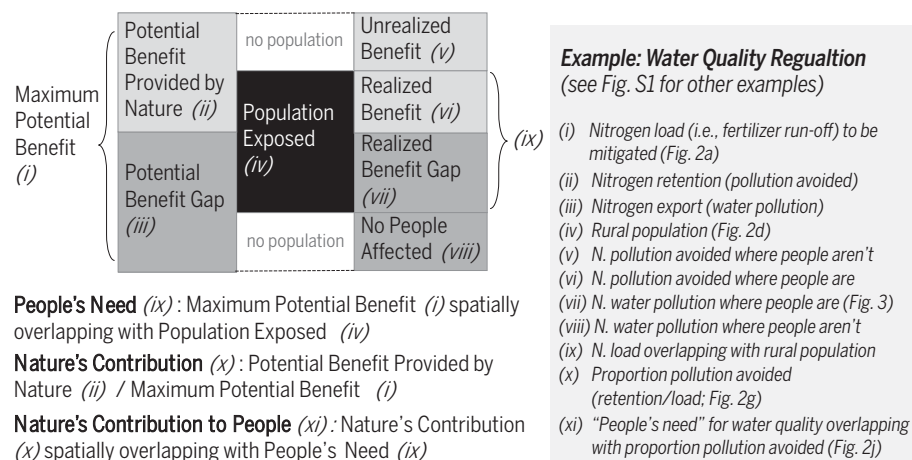


Fig. 1. Conceptual framework for calculating nature's contributions to people, with terms corresponding to Figs. 2 and 3. Maximum potential benefit (i) is based on conditions that create a human need for a benefit (see example at right, numbered corresponding to the figure). Some of this maximum potential benefit can be provided by nature (ii), and some likely cannot, leading to a potential benefit gap (iii). The maximum potential benefit, together with the population exposed (iv) to the benefit or threat, combine to form people's need (ix). In this framework, we do not consider the unrealized benefit (v) that people do not need or where no people are affected by lack of benefit (viii). The realized benefit gap (vii) is the part of people's need that is not met by nature and is often the most visible impact to people. The realized benefit (vi) is commonly considered the “ecosystem service,” which may increase simply because of greater need even without any change in nature. Thus, we consider the proportion of the maximum potential benefit provided by nature to be nature's contribution (x). Together, nature's contribution and people's need determine nature's contribution to people (xi).

¹Natural Capital Project, Woods Institute for the Environment, Stanford University, Stanford, CA 94305, USA.

²Institute on the Environment, University of Minnesota, Saint Paul, MN 55108, USA. ³Department of Natural Resource Sciences, McGill University, Sainte-Anne-de-Bellevue, Québec H9X 3V9, Canada. ⁴Basque Centre for Climate Change,

Scientific Campus of the University of the Basque Country, 48940 Leioa, Bilbao, Spain. ⁵Basque Foundation for Science, Ikerbasque, 48013 Bilbao, Spain. ⁶Centre for Development and Environment, University of Bern, 3012 Bern, Switzerland.

⁷School of Environmental and Forest Sciences, University of Washington, Seattle, WA 98195, USA. ⁸Water in the West, Woods Institute for the Environment, Stanford University, Stanford, CA 94305, USA. ⁹Kellogg Biological Station,

Department of Integrative Biology, Hickory Corners, MI 49060, USA. ¹⁰Humphrey School of Public Affairs, University of Minnesota, Minneapolis, MN 55455, USA. ¹¹German Centre for Integrative Biodiversity Research (iDiv), Martin Luther University Halle-Wittenberg, 06108 Halle, Germany. ¹²Institut für Biologie, Martin Luther University Halle-Wittenberg, 06112 Halle, Germany. ¹³CIBIO (Research Centre in Biodiversity and Genetic Resources)—InBIO (Research Network in Biodiversity and Evolutionary Biology), Universidade do Porto, 4485-661 Vairão, Portugal. ¹⁴Department of Applied Economics, University of Minnesota, Saint Paul, MN 55108, USA. ¹⁵World Wildlife Fund, San Francisco, CA 94105, USA.

¹⁶Center for Conservation Biology, Department of Biology, Stanford University, Stanford, CA 94305, USA.

*Corresponding author. Email: bchaplin@stanford.edu

time because realized benefits provided by nature could increase alongside or because of increases in maximum potential benefits (e.g., increased fertilizer runoff requiring mitigation) or population exposed (e.g., greater number of rural people affected by water contamination), although nature's contributions may remain the same. The relative proportion of nature's contribution along with people's needs, especially for the most vulnerable people, is a more useful metric than realized benefits alone when considering change across several variables at once (stressors, people, and nature) because they reveal where and when nature plays a key role in delivering benefits.

We also examine the benefits not provided by nature, or benefit gaps, and the people whose well-being may be compromised by inadequate water quality regulation, coastal risk reduction, or pollination. These benefit gaps are the outcomes people will actually face and perceive unless they are filled by other forms of capital, such as water treatment plants, sea walls, or hand pollination. Benefit gaps are what determine people's well-being, the tangible component

of nature's contributions to people, but they do not by themselves reveal the role nature plays in contributing to that well-being.

Applying this framework to operationalize nature's contributions to people, we ask two key questions. First, where is nature currently contributing most to people? And second, how many people may be affected—and where—by future changes? We examine changes from current (2015) conditions to the future (2050) under scenarios of land-use, climate, and population change according to the Shared Socioeconomic Pathways (SSP) (15). The pathways are not forecasts of the future but describe plausible major global developments (16, 17). We use three contrasting SSP narratives following the IPBES Global Assessment (4): a minimal-human-footprint vision of “sustainability” with high-intensity agriculture and urbanization, an agriculturally expansive future in “regional rivalry” due to minimal trade and high population growth, and “fossil-fueled development” with unmitigated climate change (table S1).

To address the first question, we quantify and map the overlap between people's needs

and nature's contributions (Fig. 2). We first identify places with the greatest potential for benefit: the highest pollution loads requiring retention, highest potential coastal hazards requiring mitigation, or highest crop production requiring pollination. These places are unevenly distributed for all contributions examined (Fig. 2, top row) and not always overlapping with the populations that are most reliant on those benefits (Fig. 2, second row). People's needs are greatest where the highest potential benefits overlap with the highest populations exposed. The proportion of potential benefits provided by nature (“nature's contributions”; Fig. 2, third row), regardless of whether people realize the benefits, is predictably highest where nature is most intact.

However, protection of nature will provide the greatest benefits to people where people's greatest needs coincide with nature's highest contributions (Fig. 2, bottom row, regions in black). Areas where people's needs are high and nature's contributions are low indicate benefit gaps (Fig. 2, bottom row, dark pink), manifested as pollutants not retained by

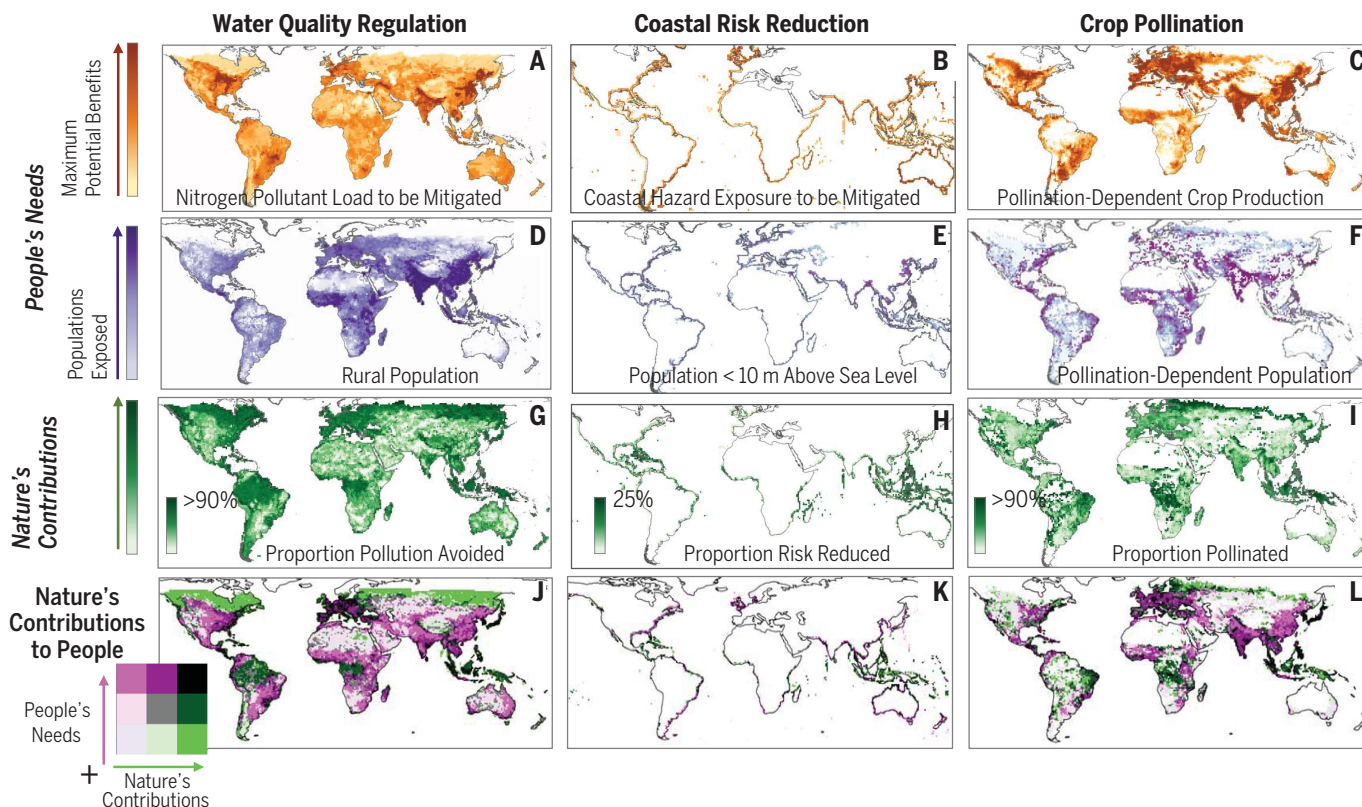
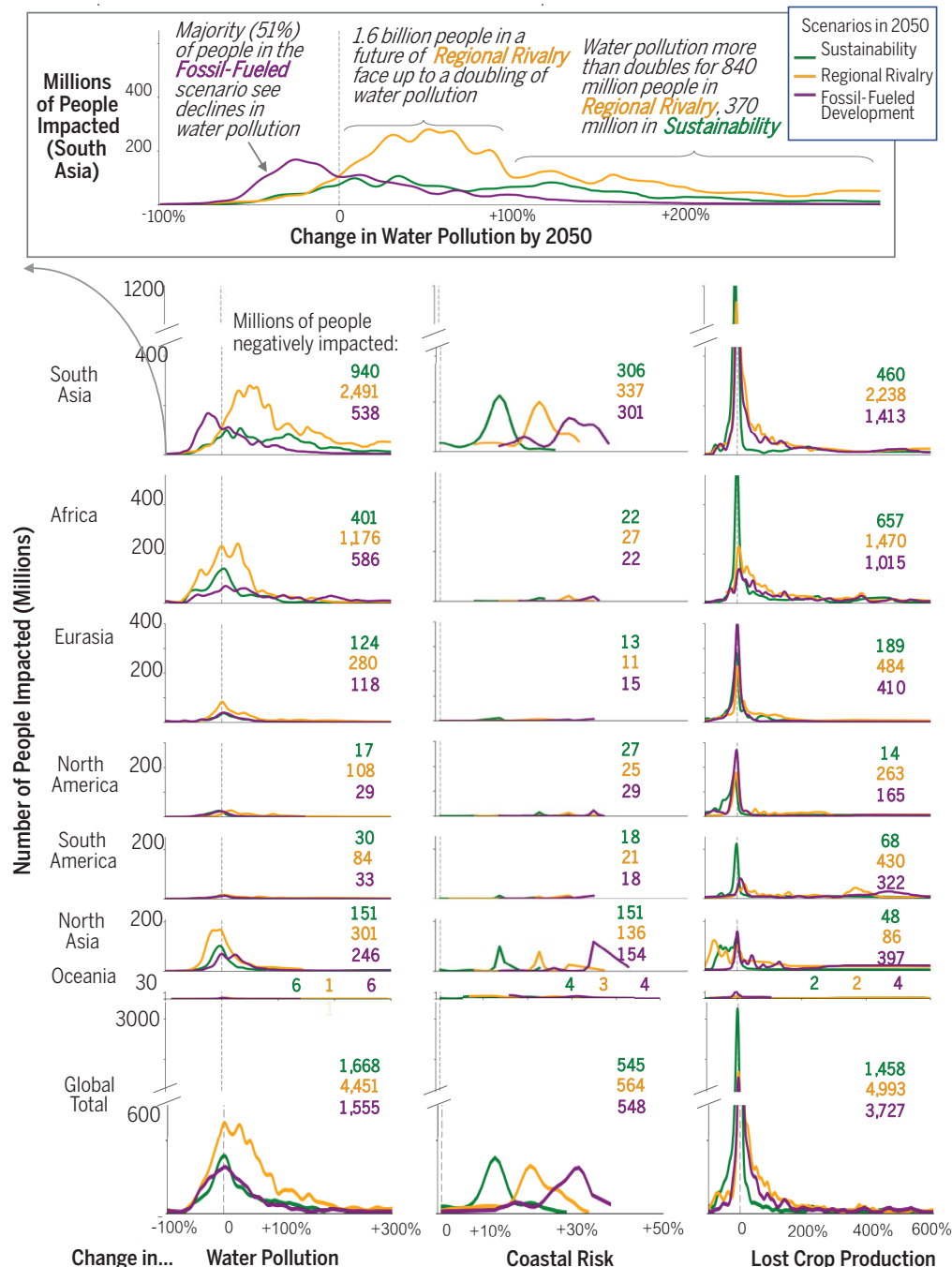


Fig. 2. Global variability in nature's contributions to people, for water quality regulation, coastal risk reduction, and crop pollination. These are quantified in terms of (A to C) (row 1) maximum potential benefits; (D to F) (row 2) population exposed to benefits or threats (rural population with presumed lower access to water treatment, coastal population falling within 0 to 10 m above mean sea level, and population whose nutritional requirements are not solely met by pollination-independent crop production within 100 km); (G to I)

(row 3) nature's contribution to providing potential benefits (proportion of pollution avoided because nitrogen was retained by vegetation, proportion of coastal risk reduced by coastal habitat, and proportion of crop pollination needs met); and (J to L) (row 4) nature's contribution to people, depicted as combined ranks of humanity's need (derived from combined ranks of pixels in maps from rows 1 and 2, in pink) and nature's contribution (ranked from row 3 in green), with black indicating the highest overlap.

Fig. 3. Future conditions result in highly uneven changes in benefit gaps (Fig. 1) across regions and scenarios. (Inset at top) A schematic of how to interpret the results, using South Asia as an example. Plots show the number of people affected (population exposed from Fig. 1) by different magnitudes of change in benefit gaps (nitrogen pollution in drinking water, risk of coastal hazards, and lost crop production due to insufficient pollination) for future scenarios of sustainability (SSP 1, RCP 2.6) in green, regional rivalry (SSP 3, RCP 6.0) in yellow, and fossil-fueled development (SSP 5, RCP 8.5) in purple. Numbers inset in each plot show millions of people negatively affected in each scenario (colored accordingly). RCP, representative concentration pathway.



vegetation before entering waterways, coastal hazards unmitigated by habitats, and crop losses from insufficient wild pollination. These mark potential opportunities for ecosystem restoration to boost nature's contributions to people, perhaps together with other investments necessary to ensure and sustain well-being.

To address the second question of where and how many people may be affected in the future, we examine the change in benefit gaps faced by different populations. Globally, up to 4.5 billion people face higher water pollution, and 5 billion may experience local losses in crop production due to insufficient pollination,

although the number of people affected in different regions could be diminished 3- to 10-fold under a sustainability trajectory (Fig. 3). Future impacts are inequitably distributed across all scenarios, with hundreds of millions of people across the globe facing benefit gaps that more than double, whereas some gaps (water pollution and crop losses) shrink for a majority of people in North Asia and North America in multiple scenarios (Fig. 3). By contrast, regardless of scenario, coastal risk increases everywhere with projected sea-level rise under climate change, affecting more than half a billion people globally by 2050. A small proportion of the

population in every region is exposed to large increases in benefit gaps, as indicated by long tails on the distributions.

Developing countries bear a disproportionate share of the impacts across scenarios. Africa and South Asia are the most disadvantaged across all scenarios for all three contributions of nature to people, with well over half the population across both regions facing higher-than-average benefit gaps, accounting for up to 2.3 billion people exposed to greater increases in water pollution, 1.7 billion facing greater additional crop losses due to insufficient pollination, and nearly 300 million facing greater increases in

coastal risk than the rest of the world (table S2). The average impacts are two to six times higher in Africa than in other regions across all scenarios and up to 10 times higher in South Asia in the sustainability scenario. Indeed, twice as many people in South Asia experience higher water pollution in this supposed sustainable future than under fossil-fueled development, likely because of the agricultural intensification in the former that results in much higher nitrogen fertilizer applications (fig. S3). However, people in this fossil-fueled future fare far worse in Africa, where the largest proportion of people globally face above-average increases in benefit gaps (table S3).

Although the models differ in their major sources of uncertainty (e.g., nitrogen loads driving variability in water quality; coastal habitat response to urbanization for coastal risk reduction; assumptions about the importance of local food supply, insensitivity to climate, and habitat quality for pollination) and lack of calibration precludes interpretation of absolute values of model outputs, relative differences between regions and scenarios as explored here have been shown in previous study to be fairly robust (12). Further work is needed to move beyond spatial overlays with population and better represent dimensions of social vulnerability and human dependence on nature, especially in terms of the availability of substitutes for natural capital (e.g., through built, technological, social, and human capital or teleconnections and trade). Yet this approach to quantifying nature's contributions to people can be made more rigorous as our data and science continue to improve.

Considering both nature's contributions and people's needs illuminates policy options. This fine-scale mapping suggests that there are relatively consolidated areas that could be targeted to close benefit gaps (e.g., in the Ganges Basin and eastern China in South Asia and in

much smaller pockets across sub-Saharan Africa; fig. S2), and examination of the natural and human dimensions of nature's contributions to people helps identify where interventions could enhance the role nature plays and where solutions should be focused on reducing people's needs. Science can provide key information for policy by connecting indicators that are measured and managed (e.g., water pollution, coastal hazards, crop losses—the benefit gaps) with the less visible yet vital roles that nature plays in filling such gaps.

The approach illustrated here is but one dimension of a much broader, systemic change needed—both in societal awareness of the importance of nature's contributions to people and in their integration into decision-making—highlighting where investments in nature may confer the greatest benefit to people, especially those who are most in need. There are a growing number of opportunities around the world for science to inform such investments, at local to national scales (1, 18–20). Ultimately, revealing nature's contributions to people, in diverse and accessible terms, is an essential step to averting the worst scenarios and transforming to a world in which both people and nature thrive.

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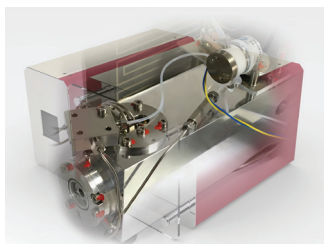
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Author contributions: All authors were involved in conceptualization and methodology and contributed to review and editing; R.C.-K., R.P.S., and C.W. conducted the formal analysis; R.C.-K. led project administration and supervision; R.P.S. led the data curation and software; C.W. and R.C.-K. led visualization; and R.C.-K., E.M.B., and G.C.D. led the writing of the original draft. **Competing interests:** The authors declare no competing interests. **Data and materials availability:** There are no restrictions on the use of materials, and all data in the analysis are available for download at <http://ipbes-natcap-ecoshard-data-for-publication.ecoshard.org> and for viewing at <http://viz.naturalcapitalproject.org/ipbes>. Code is available at Zenodo (21).

SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/366/6462/255/suppl/DC1
Materials and Methods
Figs. S1 to S3
Tables S1 to S3
References (22–77)

19 February 2019; accepted 28 August 2019
10.1126/science.aaw3372



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**Massachusetts
Institute of
Technology**

Department of Earth, Atmospheric and Planetary Sciences

Assistant Professorship in Climate-Related Sciences

The MIT Department of Earth, Atmospheric and Planetary Sciences includes a vibrant and interdisciplinary group in climate science that we seek to expand. We are especially interested in physical oceanography, atmospheric chemistry, and atmospheric dynamics, but we encourage applications from outstanding candidates in all sub disciplines of climate science. We seek candidates who use any approach or combination of approaches, including observation, theory, modeling, and experimentation. Candidates should have the potential for innovation and leadership in research and a commitment to teaching at the undergraduate and graduate levels.

Applicants must hold a Ph.D. in earth sciences or related field by the start of employment. Our intent is to hire at the assistant professor level, but more senior appointments may also be considered. Applications from women and underrepresented minorities are strongly encouraged. A complete application must include a cover letter, curriculum vitae, one- to two-page descriptions each of research and teaching plans, and three letters of recommendation.

Please explicitly commit to our department's code of conduct (<https://eapsweb.mit.edu/about/code-conduct>) in submitted cover letters.

Applications are being accepted at Academic Jobs Online: <https://academicjobsonline.org/ajob/jobs/14201>

To receive full consideration, complete applications must be received by November 1, 2019. Complete applications will be considered starting September 15 until November 1, 2019.

Search Contact: Ms. Karen Fosher, HR Administrator, EAPS, 54-924 Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139-4307, email: kfosher@mit.edu

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**Molecular
Cardiovascular
Biology**

TENURED TRACK FACULTY POSITIONS IN
MOLECULAR CARDIOVASCULAR BIOLOGY
(Assistant, Associate, Full Professor)

The Division of Molecular Cardiovascular Biology, within The Heart Institute at Cincinnati Children's is looking for qualified Ph.D., M.D. or M.D.-Ph.D. candidates with a research program that investigates or can be applied to the investigation of the molecular biology of cardiac muscle. Candidates with a skeletal muscle research focus will also be considered.

The Heart Institute at Cincinnati Children's has brought together clinical care, research and education programs, all directed at providing comprehensive care for children with heart and muscle disease and developing novel therapeutic avenues for treatment. The Heart Institute at Cincinnati Children's is one of the strongest pediatric clinical heart programs in the country.

This is an excellent opportunity to join our existing faculty of world-renowned researchers in developmental cardiac biology, pediatric and adult disease-based heart research, and skeletal muscle and muscular dystrophy biology. The successful candidate will receive a generous startup package.

Cincinnati Children's is ranked third among all Honor Roll hospitals in the 2019-20 U.S. News & World Report survey of best children's hospitals and receives the third-most NIH funds of any pediatric institution in the United States.

A letter of interest, accompanied by a complete curriculum vitae and the names of three references should be electronically sent to: Jeff.Molkentin@cchmc.org

Cincinnati Children's is an Equal Opportunity Employer.



The Department of Applied Physical Sciences (APS) invites applications for **one tenure-track Assistant Professor and one senior endowed Kenan Distinguished Professor**. Both positions are in the areas of materials science and engineering, broadly defined. The goal of APS is to bridge fundamental research with applications to address society's most challenging problems in the life sciences, soft matter, nanotechnology, the environment, and energy. APS also seeks to expand diversity in those who practice science and engineering. To achieve these goals, APS partners with all STEM and Health Affairs departments to establish team-based research, education and entrepreneurship. This hiring initiative continues a strategy to add 20 new

hires together with joint appointments from partnering departments. With our partners, APS values interdisciplinary and collaborative approaches to solve urgent societal challenges.

Candidates should have a commitment to excellence in research, interdisciplinary collaboration, extramural funding, and entrepreneurship. Duties include research, teaching, and service. Excellence in and commitment to education and mentoring at the graduate and undergraduate levels are essential qualities. The Department and University are broadly committed to equity and inclusion. It is part of our institutional mission to teach and engage a diverse community of undergraduate and graduate students, postdoctoral scholars, and faculty. We especially welcome applications from candidates who are committed to advancing these ideals. A Ph.D. in a STEM field (science, technology, engineering or math) or related fields that contribute to applied physical sciences is required by July 1, 2020.

Applications will only be accepted online (<http://unc.peopleadmin.com/postings/169918>). Applicants should submit a cover letter, a curriculum vitae, a research statement, a statement on teaching and mentorship, and 1-2 representative publications (optional). Applicants are required to identify the names, titles, email addresses and phone numbers of four professional references when applying. Each reference provider will be automatically contacted to upload their letter of support. To assure full consideration, applications should be submitted and all letters received by **November 15, 2019**. Positions are open until filled. Questions should be directed to: Chair, Applied Physical Sciences Search Committee, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3050; apssearch@unc.edu.

The University of North Carolina at Chapel Hill is an Equal Opportunity and Affirmative Action Employer. All qualified applicants will receive consideration for employment without regard to age, color, disability, gender, gender expression, gender identity, genetic information, national origin, race, religion, sex, sexual orientation, or status as a protected veteran.



**Welch Chair in
Chemistry**

The Department of Chemistry at the University of North Texas (UNT) seeks applications and nominations for an endowed Welch Chair in Chemistry, to start in the 2020-2021 academic year. The Welch Chair will lead an internationally recognized research program in any area of experimental, theoretical, or computational chemistry; contribute to the educational mission of UNT; and play a leadership role during a planned growth phase in the UNT Chemistry Department. Applicants whose research interests align with a growing emphasis area in chemical biology, or with existing strengths in computational chemistry, photochemistry, and materials, are especially welcome.

Candidates should have attained the rank of Professor or equivalent and should have a record of internationally renowned scholarly achievement as evidenced by sustained output of high-quality research publications in peer-reviewed journals. A track record of academic leadership and service to the scientific community is also desired. Generous startup and compensation package offered. For additional information and to apply formally, please visit <https://tinyurl.com/untwelchchair-apply>.

Review of applications will begin on **November 1, 2019** and will continue until the position is filled.

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- » Grant holders can apply for continued Laureate Research Grant funding, up to DKK 35 million over 7 additional years.

Novo Nordisk Foundation Young Investigator Awards

- » Individual grants up to **DKK 25 million over 7 years (EUR ~3.4 million, USD ~3.7 million)**
- » For principal investigators who have directed an independent research group for less than 7 years.
- » Award holders can apply for further funding from other Novo Nordisk Foundation grant programs.

Stage 1 application period for both calls

October 15, 2019 – November 19, 2019

To learn more, please visit: novonordiskfoundation.com



University of Pittsburgh

Alzheimer's Disease Faculty Recruitment from Multiple Disciplines

Over the next 3 years, the University of Pittsburgh, which currently ranks 4th in NIH funding to universities and research institutions, plans to hire **10-12 highly creative faculty investigators** to join a multidisciplinary team focused on determining the molecular root cause(s) of Alzheimer's disease (AD) and pursuing strategies to prevent and/or treat it. We welcome investigators who approach AD from diverse perspectives, including neurobiology, genetics, molecular and cell biology, structural biology, mitochondrial biology, neuroinflammation, immunology/virology, microbiome/metagenomics, computational biology, and other fields. We seek candidates at tenure-stream Assistant and Associate Professor levels with an MD and/or PhD or equivalent degree who have demonstrated exceptional qualifications in research, novel hypotheses and approaches to AD research, and excellent collaboration and communication skills.

With substantial financial support, we are creating a new state-of-the-art basic research center dedicated to bringing together – physically and intellectually – a critical mass of basic and translational AD investigators. We have established a marmoset colony on-site that will support translational studies in genetically engineered AD models using customized 9.4T MRI, PET-CT, and two-photon microscopy, all capable of imaging awake, behaving animals. We also support components of several NIA translational initiatives, including the mouse MODEL-AD program and AD Centers for Discovery of New Medicines. Team members will also have access to patient data, biospecimens, expertise, and resources from our Alzheimer's Disease Research Center (ADRC), brain bank, and the PET Research Center, where Pittsburgh Compound B was discovered and developed. Other key resources include the UPMC Clinical Genome Center, Pittsburgh Supercomputing Center, and School of Computing and Information, which is committed to an AD modeling initiative. Successful candidates will mentor outstanding students from multiple programs, including joint graduate programs with Carnegie Mellon University (CMU) in Computational Biology and Neural Cognition/Computation; CMU faculty are also partnering in our initiative to stop AD.

We offer competitive start-up packages, opportunities for intramural pilot funding, and one of the most livable and technologically innovative cities in the US. To be considered, please send a brief cover letter, CV, 1-page statement of AD research interests, PDFs of 2-3 recent publications, and contact information for 3 references to ADrecruit@pitt.edu.

The University of Pittsburgh is an Affirmative Action/Equal Opportunity Employer and values equality of opportunity, human dignity and diversity. EEO/AA/M/F/Vets/Disabled.



FACULTY POSITION IN GENETICS AND GENOMICS

The Department of Molecular and Cell Biology at the University of Connecticut's main campus in Storrs invites applications for a tenure-track position at the rank of Assistant or Associate professor in the area of Genetics and Genomics with an anticipated start date in August 2020. We are interested in candidates who address important questions in the broad area of genome biology using innovative and/or interdisciplinary approaches. For details on the position, qualifications, and application instructions please visit <https://academicjobsonline.org/ajo/jobs/14774> (Search #2020163). Questions about the position can be addressed to Dr. Barbara Mellone (barbara.mellone@uconn.edu)

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UNIVERSITY OF MINNESOTA

Faculty Position in Virology

The Department of Microbiology and Immunology at the University of Minnesota Medical School invites applications for a tenure-track faculty position at the Assistant Professor level. We seek a scientist working in the area of virology who will establish a competitive research program with the potential to complement the University's research strengths in host-pathogen interactions, immunity and host defense, cell biology, genetics, genomics, physiology, drug discovery, translational research, and clinical trials. The successful candidate will join a large vibrant research community spanning multiple colleges, centers, and institutes at the University of Minnesota. Further information about the department and affiliated research programs is available at www.med.umn.edu/microbiology.

Minimum qualifications: Ph.D., M.D., or equivalent in a relevant field of study, plus applicable postdoctoral or faculty experience. Review of applications will start mid-October and continue until a suitable candidate is identified. To apply, please upload a cover letter, curriculum vitae, concise summary of current and planned research, and three representative publications/pre-prints in response to Job ID 329125 at <http://www.humanresources.umn.edu/jobs>. Please also arrange to have 3 letters of recommendation sent to Stephen Rice, Ph.D., Search Committee Chair, at microbiology@umn.edu.

The University of Minnesota provides equal access to and opportunity in its programs, facilities, and employment without regard to race, color, creed, religion, national origin, gender, age, marital status, disability, public assistance status, veteran status, sexual orientation, gender identity, or gender expression.



TEMPLE HEALTH

FACULTY POSITION IN CANCER EPIGENETICS

The **Fox Chase Cancer Center** invites applicants for tenure-track faculty positions at the level of assistant professor and associate professor in the Cancer Epigenetics Program. While outstanding candidates working in all areas of epigenetics are invited to apply, we are particularly soliciting applications from candidates that are interested in the following areas:

- (1) the relationship between higher order genome organization and cancer development, progression and/or resistance;
- (2) the role chromatin has in genome stability;
- (3) the relationship between cellular signaling, chromatin modulation and cancer;
- (4) the relationship between metabolic pathways and epigenetic control;
- (5) the role of modified RNA and associated regulation in cancer;
- (6) the relationship between immune oncology and epigenetics.

Successful applicants will join a highly interactive institution that is committed to bridging basic discovery to clinical application through collaboration. Fox Chase was among the nation's first institutions to receive Comprehensive Cancer Center designation from the NCI in 1974. There are 119 research faculty that generate \$63.2 million in funding. The research faculty are surrounded by clinical colleagues that provide care for over 105,000 individuals and support >170 clinical trials, of which >60 are investigator-initiated. Two particular highlights of the center are the superb clinical pipeline that facilitates rapid translation of basic science discoveries into the clinic and the world-class core facilities that are easily accessed. Applicants interested in joining such an environment should have a Ph.D. and/or M.D. degree with an outstanding record of research productivity. The application should contain the following information - a curriculum vitae, a brief (up to two pages) statement of research interests and future goals and a list of three individuals providing letters of recommendation. Please send the application via email to Johnathan Whetstone (Johnathan.Whetstone@fccc.edu), Co-Leader, Cancer Epigenetics Program, Fox Chase Cancer Center.

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The Faculty of Physics of the Ludwig-Maximilians-Universität (LMU) in Munich is currently seeking several internationally outstanding researchers in biophysics, quantum physics or solid state physics for appointment as

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We are conducting a broad search in order to enhance and complement our research profile in these areas, which also includes two Clusters of Excellence funded within the German Excellence Strategy. The positions will be central for the further development of the Faculty and will benefit from a vibrant and collaborative research environment at the Faculty of Physics and in the wider Munich area.

For informal inquiries, please contact the Dean of the Faculty of Physics:
dekanat@physik.lmu.de

The formal advertising process will start in the next months but proactive hires will be possible as well.

Further information about the Faculty of Physics of LMU can be found at
www.en.physik.lmu.de



THE UNIVERSITY OF TEXAS AT SAN ANTONIO TENURED PROFESSOR POSITION/CHAIR, DEPARTMENT OF CHEMISTRY

The Department of Chemistry at The University of Texas at San Antonio (<https://chemistry.utsa.edu/>) invites applications and nominations for a **Tenured Professor position/Chair of the Department of Chemistry**. The Chair will lead a rapidly growing department in a Hispanic-Serving Institution that is rich in endowed positions, including two Robert A. Welch endowed positions, with nationally and internationally recognized faculty in the traditional areas of chemistry as well as interdisciplinary fields such as medicinal chemistry and materials science. The Chair will preside over the expansion of its faculty and will bolster both the graduate and undergraduate programs in chemistry as UTSA grows from its current 30,000 to 45,000 students over the next decade. This is an exciting time for the department with a wealth of opportunities as it aspires to become one of the premier chemistry departments in the country by recruiting world-leading faculty, educating and inspiring our diverse student body, and defining new chemical frontiers through transformative research endeavors.

Required qualifications of the successful candidate are a doctorate in chemistry or a related field, outstanding research and teaching records that warrant an appointment at the rank of full professor with tenure (contingent upon Board of Regents approval). The successful candidate must demonstrate their ability to work with and be sensitive to the educational needs of diverse urban populations and support the University's commitment to thrive as a Hispanic Serving Institution and a model for student success. Preferred qualifications are experience in an academic supervisory capacity/leadership position, knowledge of innovative curriculum development and implementation and leadership in professional organizations. Intellectual contributions in the areas of diversity, inclusion and prior experience teaching and mentoring students from diverse cultural backgrounds are also desirable.

Review of applications will begin on October 28, 2019 and will continue until the position is filled. Applicants should submit their CV, statements of research interests (4-page limit), teaching interests (1-page limit), and "vision and leadership" (2-page limit), all of which include discussion of the role diversity and inclusion and how it plays in an academic environment. Also include the complete contact information for three to five professional references, all to be uploaded via the STARS application system <https://jobs.utsa.edu/>. Nominations should be sent to Dr. Angela Speck, search committee chair, at angela.speck@utsa.edu.

Applicants selected for interviews must show proof that they will be eligible and qualified to work in the United States by the time of hire.

The University of Texas at San Antonio is an Affirmative Action/Equal Opportunity Employer. Women, minorities, veterans, and individuals with disabilities are encouraged to apply.

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THE UNIVERSITY OF TEXAS AT SAN ANTONIO TENURE TRACK FACULTY POSITION IN CHEMISTRY

The Department of Chemistry at The University of Texas at San Antonio (<https://chemistry.utsa.edu>) invites applications for a **Tenure-Track Assistant Professor position** beginning fall 2020. UTSA is a vibrant and growing campus with a diverse faculty focused on scholarship and the education of a multicultural community of students. Candidates must have the ability to work with and be sensitive to the educational needs of a culturally diverse urban population and be willing to contribute to UTSA's commitment to thrive as a Hispanic Serving Institution and a model for Student Success. Candidates from all subdisciplines of chemistry are welcome to apply especially those with expertise in Biochemistry or Physical Chemistry.

Candidates must have a Ph.D. in Chemistry/Biochemistry or a related field with postdoctoral experience desirable. Responsibilities will include, but are not limited to, (1) scholarly activities entailing the development of a robust, externally funded research program, (2) teaching courses to a diverse student body at the undergraduate and graduate levels, and (3) participation in institutional service activities. Screening of applications will begin November 1, 2019 and continue until the position is filled. Interested applicants can submit their application package (**as a single PDF file**) to the STARS website at <https://jobs.utsa.edu/>. The application package should include: (1) a cover letter indicating the candidate's qualifications, (2) a current curriculum vitae, and (3) a statement of research plans and teaching philosophy, including a discussion on the role diversity and inclusion plays in an academic environment (5-7 pages). Candidates should also arrange to have three letters of recommendation sent on their behalf to chemfacultysearch@utsa.edu. Those applicants selected for interviews must be able to show proof that they will be eligible and qualified to work in the United States by the time of hire.

The University of Texas at San Antonio is an Affirmative Action/Equal Opportunity Employer. Women, minorities, veterans, and individuals with disabilities are encouraged to apply.



VAGelos COLLEGE OF
PHYSICIANS AND SURGEONS

2020 Schaefer Research Scholars Program Awards

The Vagelos College of Physicians and Surgeons (VP&S) is pleased to announce the *2020 Schaefer Research Scholars Program Awards*. The *Awards*, made possible through a bequest from Dr. Ludwig Schaefer, are made annually to four research scientists who have distinguished themselves in human physiology, as broadly defined, and whose current work is of outstanding merit. The proposed research must have the potential to illuminate the field. Two awards are made to research scientists residing or working in North or South America, and two awards are made to research scientists residing or working outside North or South America. Each award consists of a \$50,000 cash prize and up to \$200,000 in direct research support.

Applications must include a cover sheet; a research proposal (one page); a research budget (not to exceed \$200,000 in total direct costs) delineated by cost category (salary, fringe, supplies, etc.) for one year (7/1/2020–6/30/2021); a curriculum vitae (not to exceed 10 pages); and a page summarizing applicant's research support. Internal candidates must obtain a nomination letter from their Department Chair. External candidates must present letters from the Dean or equivalent in their home institution as well as from the Columbia University Irving Medical Center collaborator, if applicable.

Nomination deadline: **November 12, 2019**

All materials must be submitted electronically at:
<https://www.ps.columbia.edu/schaefer>

Awards will be announced in February 2020

Tenure-Track Assistant/ Associate/Full Professor

The Division of Experimental Pathology of the Department of Pathology and Laboratory Medicine (http://rwjms.rutgers.edu/departments_institutes/pathweb/) seeks applicants for a tenure-track appointment at the Assistant/Associate/Full Professor rank. Applicants must hold a Ph.D. or M.D. degree. The successful candidate is expected to develop an independent basic research program in the field of cell biology, molecular biology, developmental biology, or immunology with strong relevance to cancer, organ-specific diseases, and translational potential. Research programs in cancer immunology, epigenetics, cell migration, morphogenesis, extracellular matrix, cytoskeletal/motor protein dynamics, and new broad-spectrum technologies are especially desirable.

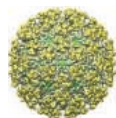
The Division of Experimental Pathology is located on the Busch Campus of Rutgers University within a core of basic science research laboratories and facilities. Rutgers University is an Equal Opportunity/Affirmative Action Employer. Applicants should electronically submit a curriculum vitae with a list of publications, reprints of no more than five representative articles, and three names of referees. Applications or inquiry should be submitted to the following email address: chen.liu@rutgers.edu.

Review of applications will begin immediately and continue until the position is filled.

Faculty Positions in Biochemistry & Molecular Biology The University of Texas Medical Branch

The University of Texas Medical Branch (UTMB Health), Galveston, Texas, seeks an outstanding faculty candidate for a tenure-track/tenured position at the rank of Assistant, Associate or Full Professor in the field of Single Molecule Biophysics in the Department of Biochemistry and Molecular Biology (<http://www.bmb.utmb.edu>). The ideal candidate will have extensive experience in using magnetic or optical tweezers, super resolution light or atomic force microscopy, to address molecular mechanisms of important biomedical problems. The candidate should be energized by opportunities to interact in a highly collaborative biomedical research community and to be an outstanding mentor to students and postdoctoral fellows. Rich opportunities exist at UTMB for interactions and affiliations with centers of scientific excellence in structural biology and molecular biophysics, mechanobiology, biodefense, molecular medicine, cancer biology, infectious diseases, environmental health, and aging (<http://www.utmb.edu/centers>). In addition to a highly collaborative environment, UTMB offers outstanding core services, including: next-generation sequencing, mass spectrometry, super resolution optical microscopy, flow cytometry, molecular biology, solution biophysics, NMR, X-ray crystallography, and cryo-electron microscopy, (see <http://www.utmb.edu/core>). Excellent opportunities for scientific interactions also exist through UTMB Health's participation in the Gulf Coast Consortia and the Keck Center for Interdisciplinary Bioscience (<http://www.gulfcoastconsortia.org>). We will accept and review applications immediately until 31 December 2019.

A very attractive recruitment package of salary, start-up funding and newly renovated space will be offered. Interested applicants should submit a curriculum vitae, a summary of research accomplishments and future goals (max 3 pages), and contact information for three references to: **Mariano A. Garcia-Blanco M.D., Ph.D.** at BMB.Recruiting@utmb.edu.



UTMB Health strives to provide equal opportunity employment without regard to race, color, national origin, sex, age, religion, disability, sexual orientation, gender identity or expression, genetic information or veteran status. As a Federal Contractor, UTMB Health takes affirmative action to hire and advance women, minorities, protected veterans and individuals with disabilities.



Center for MusculoSkeletal Research

Donald & Mary Clark Professor in Orthopaedics

The Department of Orthopaedics and the Center for Musculoskeletal Research (CMSR: www.urmc.rochester.edu/musculoskeletal-research.aspx) at the University of Rochester Medical Center (URMC: www.urmc.rochester.edu) is recruiting candidates with an international reputation in Musculoskeletal Research to assume the Donald & Mary Clark Professorship in Orthopaedics. The successful candidates will benefit from a multidisciplinary musculoskeletal research community, a vibrant graduate program and state of the art infrastructure and core facilities at the University of Rochester.

Qualified candidates should hold a tenured faculty position at the Associate Professor level or higher (or equivalent position) at a research institution, have a track record of NIH funding (or equivalent) as Principal Investigator (PI), and be active in translational areas relevant to musculoskeletal science, including but not limited to: genomics, cancer biology, bone & joint biology, and aging. The Clark Professor is also expected to lead, or work towards leading, multidisciplinary programmatic grants as PI (i.e. NIH P01), and to mentor junior faculty including clinicians.

The scientific interests pursued in the CMSR include musculoskeletal development, biomechanics and regenerative biology, skeletal pathology, bone cancer, arthritis, osteoimmunology and infections, musculoskeletal stem cell biology and metabolism, tissue engineering and targeted cell- and drug-delivery, population health, and artificial intelligence/machine learning. Interested qualified candidates should submit a CV, statement of research interests/plans, and PDFs of two or three key publications to: Jaycee_Bristol@urmc.rochester.edu. Review of applications will begin upon receipt.

The University of Rochester is an Equal Opportunity Employer and had a strong commitment to diversity and actively encourages applications from candidates from groups underrepresented in higher education.

FACULTY POSITION BACTERIAL PATHOGENESIS

The Department of Microbiology at UT Southwestern Medical Center is seeking a new faculty member in bacterial pathogenesis at the Assistant Professor (tenure track) level. Appointment rank will be commensurate with academic accomplishments and experience. The appointee will be expected to develop a front-rank, competitive, independent research program on a medically relevant bacterial pathogen(s) and/or on concepts relevant to the human microbiome. An important academic responsibility also will be the instruction and mentoring of graduate students. An attractive start-up package, including a competitive salary and generous laboratory space in a modern building, is available to conduct research within a highly dynamic environment of a leading medical microbiology department (<https://www.utsouthwestern.edu/education/medical-school/departments/microbiology>). Candidates will be considered for our \$2M Endowed Scholars (start-up) Program (<http://www.utsouthwestern.edu/education/programs/nondegree-programs/other-programs/endowed-scholars/index.html>).

Candidates should have a Ph.D. and/or M.D. degree with at least 3-4 years of postdoctoral experience and an exceptional publication record. Please send a cover letter, C.V., contact information for three letters of recommendation, and a brief summary of future research to: BacterialPathogenesisSearchCommittee@utsouthwestern.edu.

UT Southwestern Medical Center is an Affirmative Action/Equal Opportunity Employer. Women, minorities, veterans and individuals with disabilities are encouraged to apply.



TENURE TRACK FACULTY POSITION – Biochemistry

THE DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY AT THE UNIVERSITY OF MARYLAND, BALTIMORE COUNTY (UMBC) invites applications for a full-time tenure-track faculty position at the Assistant Professor level. Applicants are expected to establish a vigorous and externally funded research program in any sub-discipline of biochemistry (e.g., bioorganic, bioinorganic, bioanalytical, biophysical chemistry and chemical biology). Candidates must have an outstanding record of scientific achievement, demonstrated by publications in peer-reviewed journals. Applicants should have a PhD and postdoctoral experience in any of the sub-disciplines listed above. The selected candidate will be expected to teach primarily biochemistry courses at both the undergraduate and graduate (PhD and MS) levels. Importantly, the applicant should demonstrate an understanding of and commitment to diversity and inclusive excellence in their teaching and research programs.

Candidates should submit their applications electronically to <https://apply.interfolio.com/67120>. Application materials include curriculum vitae, a description of research plans, a statement of teaching philosophy, and a statement describing a commitment and plans to increase diversity and inclusive excellence in higher education and the discipline. The candidates should also arrange for three letters of recommendation to be submitted to <https://apply.interfolio.com/67120>. For inquiries only, please email chemsearch@umbc.edu. Review of applications will begin **November 1, 2019**, and continue until the position is filled. The appointment will commence in August 2020.

UMBC is an Equal Opportunity/Affirmative Action Employer. Applications from women, minorities, individuals with disabilities and other traditionally under-represented groups in the sciences are especially encouraged.



Eukaryotic Human Pathogen Research Department of Internal Medicine Division of Infectious Disease and International Medicine University of South Florida, Tampa campus

The Morsani College of Medicine invites applications for a tenured or tenure-track faculty position to join a developing program in eukaryotic human pathogen research (zoonotic or non-zoonotic). Candidates deciphering eukaryotic pathogen biology and/or understanding host-pathogen interaction that will ultimately lead to new diagnostics, vaccines, and therapeutics are preferred. Both basic scientists and physician scientists from diverse disciplines, including parasitology, mycology, genetics, immunology, and vector biology are encouraged to apply.

A successful candidate for this position is expected to have an established research program and grant portfolio that is transferrable to USF. Participation in graduate and/or medical student education and mentoring is also expected. The Morsani College of Medicine offers institutional salary support commensurate with experience, competitive start-up packages, dry and wet laboratory space, excellent core facilities, and a collegial atmosphere to create an environment that promotes success.

The Morsani College of Medicine is located on the Tampa campus of USF Health that includes the Colleges of Medicine, Nursing, Pharmacy, and Public Health, as well as the Moffitt Comprehensive Cancer Center and the Byrd Alzheimer's Institute. USF is ranked 41st in total research expenditures and 25th in federal research expenditures for public universities by the National Science Foundation for 2014 with extramural grants and contract activity in excess of \$450 million annually.

Applications should be submitted on line at: USF Careers (<http://www.usf.edu/about-usf/work-at-usf.aspx>) to **Job ID# 22967** and include a single PDF that includes a cover letter, curriculum vitae, research plan, and the names and contact information of five references. Review of applicants will begin on November 15, 2019 and continue until the positions are filled.



Faculty Position in Cancer Immunotherapy

The Houston Methodist Cancer Center (HMCC) is recruiting a full-time faculty member at the Assistant, Associate or Full member level (equivalent to Assistant, Associate, or Full Professor) to join our Cancer Immunotherapy Program and appropriate Houston Methodist Research Institute (HMRI) department. Researchers in this program are focused on the translation of breakthroughs emphasizing the modulation of the tumor microenvironment, host immune responses, and novel immunotherapy-based strategies for cancer. We are seeking candidates with expertise in, but not limited to, cancer vaccines, cell and gene therapy, and the tumor immune-microenvironment. Opportunities for collaboration exist with researchers in hematologic malignancies, solid tumors, nanomedicine, bioinformatics, and cancer control and population science, among other complementary disciplines.

Required: Candidates must have an MD, PhD or MD/PhD degree with established expertise in a targeted field of research, a track record of sustained and ongoing funded research program, and an outstanding record of collaborative cancer research-related activities. Preferred: Current NCI, R01 level funding. The Houston Methodist is ranked the No. 1 Hospital in Texas and nationally-ranked in nine adult specialties per *U.S. News and World Report*, 2019. HMCC is an established Center of Excellence noted for its distinction in clinical care, research, and academics. It consists of 60,000 square feet of clinical facilities dedicated for the comprehensive management and care of cancer patients. Adjacent to the HMCC clinical facilities lies HMRI. Faculty members will benefit from highly collaborative research environment, state of the art research building, infrastructure, and core services; these research facilities are housed adjacent to multi-disciplinary inpatient and outpatient cancer related clinical areas providing the opportunity for translational research. Primary appointment will be in the Houston Methodist Research Institute and the Institute of Academic Medicine, with a potential appointment at Weill Cornell Medical College upon additional approval.

To apply, please include the following with your application, and send via email to: Johnnie Atkins (jtatkins@houstonmethodist.org): (1) Cover Letter highlighting accomplishments and research interests; (2) Curriculum Vitae with publication list; (3) A list of three-four referees.



Faculty Position in Innovative Therapeutics

The Houston Methodist Cancer Center (HMCC) is recruiting full-time faculty members at the Assistant, Associate or Full member level (equivalent to Assistant, Associate, or Full Professor) to join our Innovative Therapeutics program and appropriate Houston Methodist Research Institute (HMRI) department. Researchers in this program are focused on developing the next generation cancer treatments. We are seeking candidates with expertise in, but not limited to, antibody therapeutics, small molecule drugs, gene therapy, pharmacokinetics/pharmacodynamics, and therapy resistance. They are expected to work closely with researchers in the areas of nanomedicine, cancer immunology, bioinformatics, cancer control and population science and GMP manufacturing.

Required: Candidates must have an MD, PhD, or MD/PhD degree with established expertise in a targeted field of research, a track record of sustained and ongoing funded research programs, and an outstanding record of collaborative cancer research-related activities. Preferred: Current NCI R01 level funding. The Houston Methodist is ranked the No. 1 Hospital in Texas and nationally-ranked in nine adult specialties per *U.S. News and World Report*, 2019. HMCC is an established Center of Excellence noted for its distinction in clinical care, research, and academics. It consists of 60,000 square feet of clinical facilities dedicated for the comprehensive management and care of cancer patients. Adjacent to the HMCC clinical facilities lies HMRI. Faculty members will benefit from highly collaborative research environment, state of the art research building, infrastructure, and core services; these research facilities are housed adjacent to multi-disciplinary inpatient and outpatient cancer related clinical areas providing the opportunity for translational research. Primary appointment will be in the Houston Methodist Research Institute and the Institute of Academic Medicine, with a potential appointment at Weill Cornell Medical College upon additional approval.

To apply, please include the following with your application, and send via email to: Johnnie Atkins (jtatkins@houstonmethodist.org): (1) Cover Letter highlighting accomplishments and research interests; (2) Curriculum Vitae with publication list; (3) A list of three-four referees.

UT Southwestern Medical Center

TENURE-TRACK POSITIONS

The Department of Physiology invites outstanding scientists with Ph.D., M.D., or equivalent degrees to apply for tenure-track faculty positions at the level of Assistant Professor. Candidates who bring innovative approaches to the study of any under-explored/unexplored questions broadly related to physiology are encouraged to apply. The scientific excellence of the candidates is more important than the specific area of research. These positions are part of the continuing growth of the Department at one of the country's leading academic medical centers. They will be supported by significant laboratory space, competitive salaries, state-of-the-art core facilities and exceptional start-up packages. The University of Texas Southwestern Medical Center is the scientific home to six Nobel Prize laureates and many members of the National Academy of Sciences and Institute of Medicine. UT Southwestern conducts more than 3,500 research projects annually totaling more than \$417 million. Additional information about the Department of Physiology can be found at <http://www.utsouthwestern.edu/education/medical-school/departments/physiology/index.html>.

Applicants should submit a CV, a brief statement of current and proposed research, and a summary of your two most significant publications describing the importance of the work (100-150 words each). Please arrange to have three letters of recommendation sent on his/her behalf. All items should be submitted to: <http://academicjobsonline.org/ajob/jobs/14216>. Completed applications will be reviewed starting **November 1, 2019**. You may email questions to ron.doris@utsouthwestern.edu.

UT Southwestern Medical Center is an Equal Opportunity/Affirmative Action Employer. Women, minorities, veterans and individuals with disabilities are encouraged to apply.



HARVARD
MEDICAL SCHOOL

**BLAVATNIK INSTITUTE
SYSTEMS BIOLOGY**

Assistant Professor, Department of Systems Biology

Position Description: The Department of Systems Biology at Harvard Medical School invites applications for an Assistant Professor position. We seek researchers who define and address fundamental and/or applied problems in biology or medicine, and who use quantitative experimental, computational, synthetic and/or theoretical approaches in their work.

The Department faculty have diverse backgrounds and interests in cell biology, biochemistry, physics, mathematics, chemistry, computer science, engineering and medicine. We offer a lively, family-friendly and supportive environment in which to perform interdisciplinary science, with the benefit of significant scientific and scholarly resources. We encourage team science, entrepreneurship and innovation, including in the area of mentoring and developing the next generation of scientists with diverse backgrounds and interests. We are committed to building a diverse faculty and we strongly encourage applications from, or nominations of, female and minority candidates.

The successful candidate will become a member of Harvard University's Ph.D. Program in Systems, Synthetic and Quantitative Biology, a cross-campus interdisciplinary program that attracts extraordinary graduate students.

The deadline for applications is **October 31st, 2019**. Application instructions can be found at: <https://academicpositions.harvard.edu/postings/9257>. Interviews will occur in two phases, first over video conference (early December) and then in person (weeks of 10 Feb and 24 Feb 2020).

Basic Qualifications: A Ph.D., M.D./Ph.D. or M.D. is required.

We are an Equal Opportunity Employer and all qualified applicants will receive consideration for employment without regard to race, color, religion, sex, national origin, disability status, protected veteran status, gender identity, sexual orientation, pregnancy and pregnancy-related conditions or any other characteristic protected by law.



Memorial Sloan Kettering
Cancer Center

Atomic Force Microscopist, PhD Senior Scientist - Molecular Cytology Core

The Molecular Cytology Core facility at Sloan Kettering Institute is seeking a senior research scientist to lead the planning and the execution of Atomic Force Microscopy (AFM) experiments with the members of the research and clinical laboratories at MSKCC and neighbor Institutes.

The Atomic Force Microscopist is expected to work as a member of the Core team, focusing specifically on the AFM, to continue the expansion of its use, and assist in the development and standardization of methods for the scientists. While his/her role would entail a great deal of AFM work, s/he is required to understand and work with the optical systems to facilitate and catalyze projects and cross-collaboration.

The ideal candidate should have:

- PhD degree in Biological Science with advanced AFM experience (minimally 3 years)
- Strong computational background
- Excellent communication skills and ability to work in a team environment
- Strong organization skills, flexibility in working hours and ability to multitask
- Scientific maturity, ability to understand the project goals and to assure the generation of the state-of-the-art AFM data

Please send CV, a letter outlining your interest and names/contact information of three references via email to:

Yevgeniy Romin, Molecular Cytology Core Facility, SKI rominy@mskcc.org
&

Rachel Kong, Molecular Cytology Core Facility, SKI mccf@mskcc.org

Please include "Atomic Force Microscope Position" in the subject when applying.



Memorial Sloan Kettering Cancer Center is an EO M/F/Disability/Vet Employer.



Department of Microbiology ASSISTANT/ASSOCIATE/FULL PROFESSOR POSITIONS

As part of our long-term recruiting objectives, the Department of Microbiology at the University of Washington (<https://microbiology.washington.edu>) has openings for three full-time positions at the rank of Assistant Professor tenure-track and one position at the rank of Associate Professor with tenure or Full Professor with tenure commensurate with experience. Successful candidates will perform research in any area of microbiology (bacteria, protist or viruses), including but not limited to bacterial development or behavior, virus biology or pathogenesis, microbial evolution, structural microbiology, host-microbe interactions, or microbiome studies.

We offer the opportunity to join a vibrant and diverse group of researchers at the UW, its affiliated hospitals and the wider Seattle biomedical research community. The successful candidates will find highly interactive colleagues applying a wide range of approaches to fundamental microbiology, mechanisms of microbial-host interactions, and to the development of antimicrobial strategies. The openings are 12-month service period positions in the School of Medicine with an anticipated start date of Fall 2020. In addition to research, our faculty teach outstanding trainees at the undergraduate, graduate and post-graduate levels, and engage widely in service activities.

Qualifications: Candidates for this position must hold a PhD degree in microbiology or related discipline and/or MD, (or foreign equivalent), and have a strong record of graduate and postdoctoral experience and publication. In order to be eligible for University sponsorship for an H-1B visa, graduates of foreign (non U.S.) medical schools must show successful completion of all three steps of the U.S. Medical Licensing Exam (USMLE) or equivalent as determined by the Secretary of Health and Human Services.

Application Instructions: To apply, submit a cover letter, C.V. (including a full bibliography), a concise one-page summary of research accomplishments, and a two-page summary of future research interests through our web portal: <http://apply.interfolio.com/68306>. Applicants should also arrange for three letters of recommendation from reviewers who will receive separate instructions sent automatically by Interfolio.

Applications received by **November 15, 2019** will receive a full review by the search committee. Short-listed candidates will be invited to visit the department early in 2020.



Assistant Professor of Chemistry

The Department of Chemistry and Biochemistry at Auburn University invites applications for a tenure track, nine-month position at the Assistant Professor rank. Auburn University is an institution that is both highly research-active and committed to maintaining teaching excellence as one of the nation's premier land, sea, and space grant institutions. With this position, the department is looking to fill research needs in inorganic/organometallic chemistry; candidates who can augment the department's research initiatives in chemical biology are particularly encouraged to apply. The successful candidate is expected to develop a vigorous, externally funded research program. In addition, the successful candidate must demonstrate a commitment to promoting a diverse and inclusive scholarly environment in teaching, research, mentoring, and service.

Duties also include teaching at both the undergraduate and graduate levels, thus excellent written and interpersonal communication skills are required. A Ph.D. in chemistry, biochemistry, or a closely related field and at least one year of postdoctoral experience are required at the time employment begins. The selected candidate must also meet eligibility requirements to work in the U.S. on the date of appointment (August 2020) and must be able to continue working legally for the proposed term of employment.

All applicants should submit a cover letter, curriculum vitae, a detailed statement of research plans and a two-page statement of teaching philosophy which highlights a commitment to diversity and inclusion in higher education. The applicant will also need to provide the names and contact information of three professional references. For more information about the College of Sciences and Mathematics and the Department of Chemistry and Biochemistry, please refer to our website: <http://www.auburn.edu/chemistry>. Review of applications will begin on October 31, 2019 and will continue until the position is filled.

To apply for this position, please visit:
<http://aufacultypositions.peopleadmin.com/postings/3795>

Auburn University is an EEO/Vet/Disability Employer and committed to building an inclusive and diverse community.



UNIVERSITY of MARYLAND
SCHOOL OF MEDICINE

DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY AND CENTER FOR VACCINE DEVELOPMENT FACULTY POSITIONS Baltimore, Maryland

The Department of Microbiology & Immunology and the Center for Vaccine Development and Global Health (CVD) at the University of Maryland School of Medicine are recruiting new or established investigators for multiple positions in all fields of microbiology, including immunology, bacteriology, parasitology, and virology. Expected rank is **Assistant, Associate** or full **Professor**. However, rank and tenure status are dependent on the candidate's qualifications.

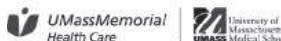
The Department and the School of Medicine have significant strengths in microbial pathogenesis, vaccine development, HIV biology, genome sciences, inflammation, innate and adaptive immunity, clinical infectious diseases and international research and training. The Department offers excellent laboratory facilities, competitive salary and startup packages, and access to numerous core facilities including state-of-the-art BSL3 and ABSL3 facilities. The CVD comprises a multi-disciplinary team of faculty whose primary mission is to develop and implement vaccines and other interventions to reduce the burden of infection throughout the world.

We are particularly interested in candidates with interests or experience in either vaccine development for viral pathogens or immune responses to Plasmodium infections. Successful candidates are expected to maintain active research programs and participate in department teaching and service opportunities. Interested applicants are invited to submit their application using the following link: <https://umb.taleo.net/careersection/jobdetail.ftl?job=190000U7&lang=en>

Consideration of candidates will begin upon receipt of applications and will continue until a suitable candidate is identified. For questions please contact Dr. William Jackson at Wjackson@som.umaryland.edu

The University of Maryland, Baltimore and the University of Maryland School of Medicine are Equal Opportunity/Affirmative Action Employers. All qualified applicants will receive consideration for employment without regard to sex, gender identity, sexual orientation, race, color, religion, national origin, disability, protected Veteran status, age, or any other characteristic protected by law or policy. We value diversity and how it enriches our academic and scientific community and strive toward cultivating an inclusive environment that supports all employees.

Diabetes CENTER OF EXCELLENCE



FACULTY POSITION

The Diabetes Center of Excellence at the University of Massachusetts Medical School invites applications for a SENIOR TENURED or JUNIOR TENURE-TRACK basic science or clinical investigator faculty position. The Diabetes Center of Excellence currently consists of basic and physician scientists representing a broad range of disciplines in the biomedical and clinical sciences, with members from several Medical School Departments and Programs working together as central New England's leading center for diabetes clinical care, innovation, and discovery. The Center occupies state-of-the-art research space on an expanding Medical School campus. Basic investigators benefit from core facilities for deep sequencing, proteomics, genotyping, fluorescence-activated cell sorting, digital imaging/confocal microscopy, genomics/bioinformatics, transgenic/knockout mice, and mouse metabolic phenotyping. Clinical and community investigators benefit from infrastructure for recruitment and retention of research participants, measurement, behavioral and nutritional intervention resources and facilities, and academic-community research partnerships. Adult and pediatric clinical efforts of the Diabetes Center are housed in a wonderful Ambulatory Clinical Care building designed so that patients can receive appropriate retinal photos, foot care, diabetes education, and other routine medical care at one location. The position will be highly competitive with regard to start-up funds, laboratory space (if needed) and salary. The Center seeks an individual of outstanding research potential relating to diabetes pathophysiology or treatment.

Applicants should send curriculum vitae, statement of research interests, and names and addresses of three references to: **David M. Harlan, M.D and Dale Greiner, Ph.D., Co-Directors, Diabetes Center of Excellence, Search Committee Co-Chairs**, c/o Patricia Cannon, 55 Lake Avenue North, Albert Sherman Center, Room AS7.2049, Worcester, MA 01605; Patricia.cannon@umassmed.edu; 508.856.3800.

As an Equal Opportunity and Affirmative Action Employer, UMMS recognizes the power of a diverse community and encourages applications from individuals with varied experiences, perspectives, and backgrounds.



Faculty Positions in Neuroscience

The Neuroscience Institute at Georgia State University (GSU) invites applications for up to two tenure track positions at the rank of Assistant Professor. Preference will be given to individuals using state-of-the-art cross disciplinary approaches incorporating molecular, genetic/genomic, computational, or quantitative methods. Candidates must hold a Ph.D. or M.D./Ph.D. with evidence of excellence in research and the ability to establish a vigorous, externally funded research program while contributing to the Neuroscience Institute's graduate and undergraduate teaching. The ideal research program would synergize with existing strengths in the Neuroscience Institute (see <http://neuroscience.gsu.edu>) and GSU's interdisciplinary University Research Centers (<https://research.gsu.edu/about/>). Georgia State is an enterprising R-1 university located in the heart of downtown Atlanta, a vibrant international city in the Southeastern U.S. GSU enrolls and graduates one of the most diverse student bodies in the nation. The Institute is committed to serving this student body and advancing innovative research by building a diverse faculty.

Applicants should provide a full curriculum vita, names and contact information for three references, and a statement of research plans and teaching philosophy (5-page limit) that includes their views and experience related to the Institute's goal of serving a diverse student body in its educational activities. Applications can be submitted either electronically in PDF format to nisearchcommittee@gsu.edu or in hard copy to: Chair of the Neuroscience Institute Faculty Search Committee, Neuroscience Institute, PO Box 5030, Georgia State University, Atlanta, GA 30302-50310. Review of applications will begin **November 1, 2019** and continue until the position is filled.

Georgia State University is an Equal Opportunity/Affirmative Action Employer and does not discriminate against applicants due to race, ethnicity, gender, veteran status, or on the basis of disability or any other federal, state or local protected class.



INDIANA UNIVERSITY
MOLECULAR AND CELLULAR
BIOCHEMISTRY DEPARTMENT
College of Arts and Sciences
Bloomington

ASSISTANT PROFESSORS IN CHROMOSOME BIOLOGY AND CRYO-ELECTRON MICROSCOPY

The Department of Molecular and Cellular Biochemistry (MCB) at Indiana University invites applications for two positions beginning

August 2020 at the rank of Assistant Professor. The first position is for biochemists who employ structural or single-molecule approaches to study chromosome biology. The second position is for candidates in all areas of biochemistry who use cryo-electron microscopy as a primary tool and the position holder will be affiliated with Indiana University's emerging Center for Chemical Biology and Biotherapeutics (C2B2) organized under the Precision Health Initiative (PHI); <https://grandchallenges.iu.edu/precision-health/index.html> in collaboration with the Indiana University School of Medicine. A Ph.D. in biochemistry, chemistry or a related field with relevant postdoctoral experience are required. Successful candidates will be expected to develop or already have a visible, externally funded research program. Primary teaching assignments will involve undergraduate and graduate level biochemistry or related courses in the molecular life sciences.

Interested candidates for Chromosome Biology position should submit their application at (<https://indiana.peopleadmin.com/postings/8399>).

Interested candidates for Cryo-Electron Microscopy position should submit their application at (<https://indiana.peopleadmin.com/postings/8483>).

A completed application includes a curriculum vitae, a summary of future research plans. The applicant should also arrange the submission of **four letters** of recommendations addressed to Chair, Faculty Search Committee. Questions regarding the position or application process can be directed to: Suzanne Schwartz (mcbdept@indiana.edu). Applications received by November 15, 2019 will receive full consideration; later applicants will also be considered until the position is filled. The College of Arts and Sciences is committed to building and supporting a diverse, inclusive, and equitable community of students and scholars.

Indiana University is an Equal Employment and Affirmative Action Employer and a provider of ADA services. All qualified applicants will receive consideration for employment without regard to age, ethnicity, color, race, religion, sex, sexual orientation, gender identity or expression, genetic information, marital status, national origin, disability status, or protected veteran status.



PennState
Eberly College of Science



Eberly Research Fellows at Penn State University

The Eberly College of Science at Penn State University invites nominees for the Eberly Research Fellowship program. Eberly Fellowships are designed to attract exceptional early career scientists to Penn State to enhance their career goals in the vibrant, highly collaborative environment of the Eberly College of Science and the broader STEM community of Penn State University. The Eberly College of Science which includes the Departments of Astronomy & Astrophysics, Biology, Biochemistry & Molecular Biology, Chemistry, Mathematics, Physics, and Statistics, ranks in the top 10 universities in the U.S. and has annual research expenditures exceeding \$127M. Each of the seven departments is expecting to appoint one or more Eberly Fellows. Nominations for early career scientists with exceptional promise in basic research in physics, chemistry, biology, molecular biology, astronomy, mathematics, and statistics and/or applied research in health, energy, materials, or the environment are encouraged. Interdisciplinary as well as traditional disciplinary research is encouraged. Fellows who wish to also gain training and experience in teaching may elect to receive mentored teaching experience. Eberly Fellow advisors must hold their primary appointment in one of the seven departments of the Eberly College of Science. Co-advisors and cross-disciplinary research are also supported.

Eligibility and appointment

Applicants must be a current doctoral student or have received a doctoral degree in science, statistics, or mathematics within the past three years. Current doctoral students must have their doctoral degree prior to the start of their fellowship. Current doctoral students and postdoctoral fellows at Penn State are not eligible. Eberly Research Fellowships may be held for up to 3 years (with an initial appointment of 1 year and up to 2 additional annual appointments conditional on progress, funding, and eligibility). Fellows will receive a stipend of \$65,000 and \$5,000 per year in discretionary funds, for travel and other research expenses.

Nomination and applications

Nominations will be accepted from past and/or current faculty advisors, graduate program chairs, department chairs, or others who can attest to the nominee's potential as a scientist. Nominations should include the nominees CV. Nominations of women and under-represented minorities are strongly encouraged. The review of nominations will begin on November 1, 2019. Nominations should be sent to researchfellows@psu.edu.

The Departmental Eberly Research Fellowship Selection Committees will select the nominees to be invited to submit applications which will be reviewed beginning on December 16, 2019. Applications will include (1) a biographical sketch – including publications, accepted, and submitted manuscripts, (2) three letters of reference including one from the doctoral advisor, (3) research statement summarizing research accomplishments and research that you intend to pursue at Penn State, (4) names of one or more potential faculty advisors among the faculty in the seven departments of the Eberly College of Science, Penn State University.

CAMPUS SECURITY CRIME STATISTICS: For more about safety at Penn State, and to review the Annual Security Report which contains information about crime statistics and other safety and security matters, please go to <http://www.police.psu.edu/clery/>, which will also provide you with detail on how to request a hard copy of the Annual Security Report.

Penn State is an equal opportunity, affirmative action employer, and is committed to providing employment opportunities to all qualified applicants without regard to race, color, religion, age, sex, sexual orientation, gender identity, national origin, disability or protected veteran status. U.Ed. SCI 20-14

WAYNE STATE UNIVERSITY

Tenure/Tenure Track Faculty Position in Biochemistry

The Department of Biological Sciences at Wayne State University invites applications for a tenure-track opening for a researcher studying **Biochemistry** with a focus on metabolomics. Rank will be dependent upon qualifications. Preference will be given to candidates doing innovative research in areas complementing existing departmental strengths in systems biology, development, the biochemistry of disease, evolution, host/pathogen interactions or chromatin and gene expression. Applicants must have a Ph.D. degree, postdoctoral experience and an outstanding record of research achievement. Successful applicants are expected to establish and maintain a vigorous, externally funded research program, participate in graduate and undergraduate education and perform service at the departmental, college and university level. We offer state-of-the-art research facilities and highly competitive start-up packages.

Wayne State University is a premier, public, urban research university located in the heart of Detroit where students from all backgrounds are offered a rich, high quality education. Our deep-rooted commitment to excellence, collaboration, integrity, diversity and inclusion creates exceptional educational opportunities preparing students for success in a diverse, global society. WSU encourages applications from women, people of color and other underrepresented people. WSU is an affirmative action/equal opportunity employer.

Please submit a cover letter, curriculum vitae, a diversity statement and a 2-page statement of research plans at jobs.wayne.edu (posting # 044663) by **November 20, 2019** for full consideration. Three letters of reference should be sent to: Faculty Search Committee, Department of Biological Sciences, Wayne State University, Detroit MI 48202 (Ariel.Delegol@wayne.edu). Applications will be considered only when all materials have been received.



Faculty Positions in Pharmacology and Nutritional Sciences

The Department of Pharmacology and Nutritional Sciences in the College of Medicine is seeking faculty candidates at the tenure-eligible or tenured ranks. Preferred candidates should have an extramurally funded research program, or for junior candidates, high promise of securing extramural funding, on basic and/or translational research in diabetes/obesity or related comorbid conditions. This research expansion aligns with our campus-wide focus on health disparities and our newly consolidated Healthy Kentucky Research Group structured to bring diverse groups of experts together to solve the most pressing healthcare problems in our region and beyond.

Areas of strength in the department, the broader research group and centers include basic, translational, and clinical research on mechanisms of diabetes/obesity and associated diseases including cardiovascular and fatty liver diseases, cancers and dementias/aging. Candidates should have a PhD, MD or equivalent degree, a successful postdoctoral experience, and for more senior applicants, a history of sustained productivity as evidenced by publications, grant funding, and mentorship. Successful candidates will be offered highly competitive start-up packages. Please note that faculty are expected to participate in the department's academic programs. More information on the department can be found at <https://pharmns.med.uky.edu/>

The University of Kentucky has state-of-the-art research facilities and the department is top-ranked among public and private universities. UK has also been recognized by Forbes and The Chronicle of Higher Education as a "Best Employer" in several categories.

Applicants should submit a cover letter, CV, brief description of research interests, copies of 2-3 major publications and contact information for 3 references via our online employment application system at either <http://ukjobs.uky.edu/postings/251585> or <http://ukjobs.uky.edu/postings/251606>. Review of applications will begin **November 15, 2019**, or shortly thereafter, and continue until positions are filled.

The University of Kentucky is an Equal Opportunity University that values diversity and inclusion. Minorities, veterans, women, individuals with disabilities, and members of other underrepresented groups are strongly encouraged to apply.



SCHOOL OF MEDICINE
CASE WESTERN RESERVE
UNIVERSITY

Assistant or Associate Professor Department of Biochemistry

Case Western Reserve University, Department of Biochemistry invites applications for tenure-track faculty or tenured faculty positions. While applications from individuals with interests in any area of biochemistry are requested, areas of specific interest include metabolism/metabolomics, RNA biology, stress response signaling, and chromatin biology as they pertain to fundamental aspects of human diseases such as cancer biology.

Minimum Qualifications: Successful candidates are expected to develop innovative and externally funded research programs, participate in departmental education programs, and contribute to service opportunities. Candidates should have a PhD and/or MD with a strong record of research accomplishments. Candidates at the Assistant Professor level will have significant postdoctoral research experience with the potential for developing an extramurally funded and nationally recognized research program, while candidates at the Associate Professor level will have a funded and nationally/internationally recognized research program.

The Department of Biochemistry offers outstanding startup packages, modern laboratory space, excellent core facilities and a highly collaborative environment.

How to Apply: Review of applicants will begin on November 1, 2019. Send a letter of application, curriculum vitae, description of research interests, and contact information for 3 references to: Biochemistry faculty search committee, Department of Biochemistry, CWRU, 10900 Euclid Avenue, Cleveland, OH 44106; biocfacsearch@case.edu. Please arrange for references to send recommendation letters separately to the same address.

Case Western Reserve University is committed to Equal Opportunity and Diversity. Women, veterans, members of underrepresented minority groups, and individuals with disabilities are encouraged to apply.

Case Western Reserve University provides reasonable accommodations to applicants with disabilities. Applicants requiring a reasonable accommodation for any part of the application and hiring process should contact the Office for Inclusion, Diversity and Equal Opportunity at 216.368.8877 to request a reasonable accommodation. Determinations as to granting reasonable accommodations for any applicant will be made on a case-by-case basis.



HARVARD MEDICAL SCHOOL

DEPARTMENT OF IMMUNOLOGY

The new Department of Immunology at Harvard Medical School invites applications for a tenure-track position at the rank of Assistant Professor. We seek an outstanding scientist performing basic and/or translational research in any area of innate or adaptive immunity. This position offers outstanding scholarly and scientific resources in a collegial and collaborative department with strong ties to related departments throughout Harvard University, the Harvard-affiliated teaching hospitals, and the Boston immunology community. The position offers the opportunity to teach exceptional graduate and medical students with strong interests in immunology. The research space will be located in a modern and central research building at Harvard Medical School. Candidates must have a PhD, MD, or an equivalent graduate degree. For consideration please upload to the website below a cover letter, a C.V. (including list of publications), and a concise summary of research accomplishments and future interests. Three letters of recommendation will also be required. Applications should be submitted by **November 30, 2019**, <https://academicpositions.harvard.edu/postings/9268>. Applications received after this date are not guaranteed a review.

Contact Information:

David Lucas, PhD
Harvard Medical School
77 Avenue Louis Pasteur, NRB 1030
Boston, MA 02115
david_lucas@hms.harvard.edu

We are an Equal Opportunity Employer and all qualified applicants will receive consideration for employment without regard to race, color, religion, sex, national origin, disability status, protected veteran status, gender identity, sexual orientation, pregnancy and pregnancy-related conditions or any other characteristic protected by law.



UNIVERSITY OF NOTRE DAME

Faculty Position in Rare Diseases

The Department of Biological Sciences at the University of Notre Dame invites applications for a tenure-track assistant professor position in rare diseases. This is an endowed, junior chair position affiliated with the Boler-Parseghian Center for Rare and Neglected Diseases (<https://crnd.nd.edu>).

We seek outstanding candidates who conduct cutting-edge biomedical research. Prospective candidates must have a Ph.D. degree and postdoctoral experience with demonstrated research accomplishments. Candidates with research programs in rare diseases including, but not limited to, those in neurobiology, cancer and immunology are encouraged to apply.

Hiring is anticipated at the Assistant Professor level. The successful candidate is expected to develop an internationally recognized research program in rare diseases. They will have the opportunity to actively engage in the Center for Rare and Neglected Disease's private-public-partnerships with the pharmaceutical industry, as well as innovative research training that includes mentored graduate and undergraduate scholarship in rare diseases, and other Center initiatives of patient-centered research. The successful candidate will engage in teaching at the graduate and undergraduate levels.

A diverse community of Notre Dame researchers is supported by numerous Centers and Institutes including, the Center for Stem Cells and Regenerative Medicine, Harper Cancer Research Institute, Eck Institute for Global Health, Keck Center for Transgene Research, Center for the Study of Biocomplexity, and Center for Zebrafish Research. Additional facilities to support research include the AAALAC-accredited Freimann Animal Facility, Integrated Imaging Facility, and cores in Structural Biology, Bioinformatics, Genomics and Proteomics.

The position includes an attractive salary, competitive start-up package, and laboratory space tailored to the applicant's research needs. Information on center, department and other college faculty and facilities can be found at <http://biology.nd.edu>, <http://science.nd.edu> and <https://crnd.nd.edu>. Opportunities also exist for collaboration with faculties at the adjoining Indiana University School of Medicine-South Bend.

The University of Notre Dame seeks to attract, develop, and retain the highest quality faculty. The University is an Equal Opportunity Employer committed to building a culturally diverse and inclusive community and supports the needs of dual career couples. We strongly encourage applications from female and minority candidates.

Review of applications will commence on **November 1, 2019** and continue until suitable candidates are identified. Qualified individuals should send in pdf format, a cover letter, curriculum vitae, separate statements of research and teaching interests, and three letters of reference to: apply.interfolio.com/68812. Queries may be directed to raredisease@nd.edu.

The University of Notre Dame, an international Catholic research university, is an Equal Opportunity Employer.



UNIVERSITY OF NOTRE DAME

Open Rank Tenure-Track Faculty Positions in Vector Biology

The Department of Biological Sciences seeks to recruit tenure-track faculty at an academic rank of Assistant, Associate or Full Professor who study the biology of arthropod vectors of human disease or vector-pathogen-host interactions. Individuals with expertise in ecology, behavior, physiology, or evolutionary, population and functional genomics, particularly those with cross-disciplinary and field-based research programs, are encouraged to apply.

The successful candidates will be highly productive with an established, or strong potential to establish, a vigorous externally funded research program that complements and synergizes with others in the department and across the University studying pathogen genomics, pathogenesis, disease ecology, epidemiology, and climate change.

New faculty will contribute to the undergraduate and graduate teaching mission of the Department of Biological Sciences and join an integrative and collaborative research community with expertise that spans the breadth of the life sciences. Several faculty have active research partnerships with international field sites and associated research partners, including in Africa, Latin America, Asia, and the South Pacific. Department faculty have access to state-of-the-art genomics, bioinformatics, computing, mass spectrometry, proteomics, and imaging cores, specialized BSL-3 containment laboratories, insect rearing and research facilities, and an AAALAC-accredited animal facility. Information on department and other college faculty and facilities can be found at <http://biology.nd.edu> and <http://science.nd.edu>. Opportunities also exist for collaboration with faculty at the adjoining Indiana University School of Medicine-South Bend.

The University of Notre Dame seeks to attract, develop, and retain the highest quality faculty. The University is an Equal Opportunity Employer committed to building a culturally diverse and inclusive community and supports the needs of dual career couples. We strongly encourage applications from female and minority candidates.

Review of applications will commence on **November 27, 2019** and will continue until suitable candidates are identified. Applicants should submit in PDF format, a cover letter, curriculum vitae, names and contact information of three professional references, 2-page statement of research interests and future research plans, and 2-page statement of teaching philosophy, approach and experiences, to: apply.interfolio.com/68836. Interested individuals are welcome to contact the search chair, Professor Nora Besansky, at nbesansky@nd.edu.

The University of Notre Dame, an international Catholic research university, is an Equal Opportunity Employer.

CALL FOR NOMINATIONS

Mechthild Esser Nemmers Prize in Medical Science



Northwestern University Feinberg School of Medicine invites nominations for the Mechthild Esser Nemmers Prize in Medical Science. This prize includes a cash award of \$200,000. Candidacy for the 2020 Nemmers Prize is open to physician-scientists whose body of research exhibits outstanding achievement in their disciplines as demonstrated by works of lasting significance. Individuals of all nationalities and institutional affiliations are eligible except current or recent members of the Northwestern University faculty and recipients of the Nobel Prize.

The 2020 recipient of the Nemmers Prize will deliver a public lecture and participate in other scholarly activities at Northwestern University. Nominations for the Prize will be accepted until November 1, 2019.

For further information, and to submit a nomination, please visit
feinberg.northwestern.edu/nemmers



The Nemmers Prize in Medical Science
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香港中文大學(深圳)
The Chinese University of Hong Kong, Shenzhen

**Professor/Associate Professor/Assistant Professor/Lecturer
The Chinese University of Hong Kong, Shenzhen**

The School of Life and Health Sciences (LHS) at The Chinese University of Hong Kong, Shenzhen (CUHK-Shenzhen) invites applications for full-time faculty positions at the indicated academic ranks. Major research areas include, but are not limited to, biomedical engineering, computational biology and bioinformatics, structural biology, chemical biology, neuroscience, precision and regenerative medicine, and cancer immunotherapy. Qualified candidates must have an earned doctoral degree in biomedical sciences, postdoctoral training and work experience in related research areas. Applicants for Professors and Associate Professors should have an established research program with funding history, teaching experiences, and an excellent publication record. Lecturer and Senior Lecturer positions are available for candidates with biomedical teaching experience.

Established in 2014, CUHK-Shenzhen is a research-intensive university that inherits the fine academic traditions of The Chinese University of Hong Kong. The University adopts a tenure-track system for Assistant Professors and above. English is the main language for course instructions, and the students receive degrees of The Chinese University of Hong Kong. LHS was established in November 2018 and is the fourth school in CUHK-Shenzhen that offers bachelor, master and doctoral degrees. Please visit lhs.cuhk.edu.cn for additional descriptions of the teaching and research programs at LHS. Interested applicants should submit their curriculum vitae, teaching statements and research descriptions to: <http://academicrecruit.cuhk.edu.cn/lhs>. Applications will be reviewed on a rolling basis until the positions at the respective ranks are filled.

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家人在那头。

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By Brittany L. Uhlorn

Making peace with imperfection

“I can eat 200 grams of sweet potato, 4 ounces of ground turkey, and 90 grams of lettuce for lunch,” I thought to myself. “I have to run 2 miles after my workout to make up for that chocolate chip I ate yesterday, and I need to check my weight tomorrow to make sure I didn’t gain anything overnight.” “Brittany? Brittany! Any suggestions for troubleshooting your labmate’s experiment?” My graduate school adviser was trying to get my attention during lab meeting—but there I was again, meticulously planning meals and obsessing over exercise, oblivious to the eating disorder I had developed to cope with the stress and anxiety that accompanied my quest for perfection.

It started 2 years into my Ph.D., after my comprehensive exam. I had always succeeded academically, aiming for a pedestal defined by others’ standards. As I stepped in front of my exam committee, I was eager to dazzle. But I was told my hypothesis wasn’t valid. I was chastised for lack of creativity. My simplistic sketch of the Golgi drew derisive snickers. Even though I passed, I believed I was a failure.

Suddenly, for the first time, I felt completely out of control. The result was debilitating stress and anxiety. Up to that point, I had never denied myself food, and I exercised for fun—not out of obligation. But the Instagram fitness influencer craze lured me into thinking I could take back control of my life by perfecting my physique.

What began as a seemingly harmless “lifestyle change” turned into orthorexia nervosa, an unhealthy obsession with supposedly healthy eating and exercise (but not body weight, in contrast to anorexia or bulimia). I spent my days using my research skills to methodically plan a restrictive diet complemented by a strict exercise routine. During lab meeting, instead of paying attention to the research being presented, my mind was fixated on the ideal ratio of carbs, fats, and protein. I spent more time weighing rice cakes and spinach than planning the experiments I needed to do to finish my manuscript. I often turned down social gatherings so that I could eat “safe” meals at home.

I had gained a false sense of control and suppressed my emotions, but the underlying problems were still there—and getting worse. Over 9 months, as my stress and anxiety increased, orthorexia developed into anorexia. I compulsively stepped on the scale multiple times a day and spat out food after chewing. I ate from the smallest dishes to



“There I was again, meticulously planning meals and obsessing over exercise.”

give the illusion of a full plate and denied my audibly growling stomach the food it needed. I bruised from sitting in chairs and would black out upon standing. I couldn’t sleep, and I jeopardized my relationships. I became a walking skeleton.

My family and adviser asked whether I was OK and suggested time away from lab, but my disordered brain made excuses for how I was feeling and behaving. I honestly believed I was thriving.

It wasn’t until I stumbled across an article about disordered eating as a coping mechanism for mental health issues that I put a name to the problem. After 2 months of spiraling deeper into my disorder, thinking I could “fix” it on my own, I finally sought the help of a therapist.

I learned that my eating disorder had nothing to do with food, exercise,

or weight; instead, it was about my desire to be exceptional. Through weekly sessions, I unpacked 25 years of needing to be perfect. I learned tools to acknowledge and process my feelings, instead of numbing them with calorie restriction and intense workouts.

Still, in times of stress the disordered part of my brain desperately clung to food deprivation. Restoring my weight took more than a year, as well as the help of a dietitian. Gradually I redefined food and exercise as sustenance for body and mind, not tools for suppressing uncomfortable feelings.

Now, a year and a half into my recovery, I am entering my final year of graduate school with a completely different mindset about the person I want—rather than need—to be. Yes, I still want to succeed. But I don’t want to be perfect. ■

Brittany L. Uhlorn is a Ph.D. candidate at the University of Arizona in Tucson. Send your career story to SciCareerEditor@aaas.org.

ILLUSTRATION: ROBERT NEUBECKER